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14. ABSTRACT

This project is to use a unique mouse model to study the interaction of reproductive factors and genetic mutations in the development of ovarian cancer. Ovarian cancer often develops in women of peri-menopausal age. We found that the germ cell deficient Wv mice mimics postmenopausal biology and develop benign ovarian tumors. We plan to test the hypothesis that a synergy exists between oncogenic mutations such as p53, pten, or p27kip1 and postmenopausal biology in ovarian cancer development. In the first year of the project, we completed Aim 1, the study of ovarian tumor phenotypes in mice of compound genotypes. We found that crossing of Wv mice into mutant p53, pten, or p27 background did not lead to a malignant tumor phenotype. Instead, the mutants rescue ovarian germ cells, a very interesting finding. The ovarian surface epithelia in these compound mutant mice develop unique lesions with peculiar morphology, which are undergoing analysis as planned in Aim 2. In future study, we plan to use flox-p53 mutant mice to create mutation only in ovarian surface epithelial but not in germ cells (Aim 3). In sum, the project progresses as planned. We have layered the basis and are posed to further advance.

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1. Introduction

This project is to use a unique Wv mouse model to study the interaction of reproductive factors and genetic mutations in the development of ovarian cancer. Ovarian cancer often develops in women of peri-menopausal age, when ovulation ceases but gonadotropin levels are increased. We found that the germ cell deficient Wv mice mimics postmenopausal biology and develop benign ovarian tumors. We plan to test the hypothesis that the synergy between oncogenic mutations and postmenopausal biology can be revealed by combining the germ-cell deficient phenotype of the Wv/Wv mice and genetic alterations such as p53, pten, or p27kip1, which are found in human ovarian cancer.

2. Body: Research Progress

In the first year of the project, we completed **Aim 1**/*Task 1., t*o determine the optimal genetic changes that synergize with the Wv genotype for the development of malignant ovarian tumors in mice (Months 1-12). We crossed and obtained several female Wv mice with additional p53 (-/-), p27 (-/-), or pten (+/-) mutation and analyzed the ovaries for tumor phenotype. We found that crossing of Wv mice into mutant p53, pten, or p27 background did not lead to development of large tumors or a malignant tumor phenotype. Instead, the additional mutations rescue ovarian germ cells, a very interesting finding. At 3 months of age, the wildtype ovary contains many germ cells and follicles that are indicated by positive staining with marker PGC7 (**Figure 1**). The Wv/Wv ovary shows infiltration with epithelial derived tumor cells and contains no germ cells or follicles (**Figure 1**). As shown in a representative figure (**Figure 2**), an ovary of Wv/Wv:p53 (-/-) mouse showed the presence of follicles, comparing to those wildtype and Wv/Wv littermates (**Figure 1**). Deletion of p53 in the Wv/Wv:p53 (-/-) ovary results in the absence of tumor and the presence of several germ cells/follicles in the section observed (**Figure 2)**. This finding suggests p53 is very important for the survival and lifespan of ovarian germ cells and follicles. Also, the finding suggests that the depletion of follicles is key causal factor for the ovarian tumor phenotype.

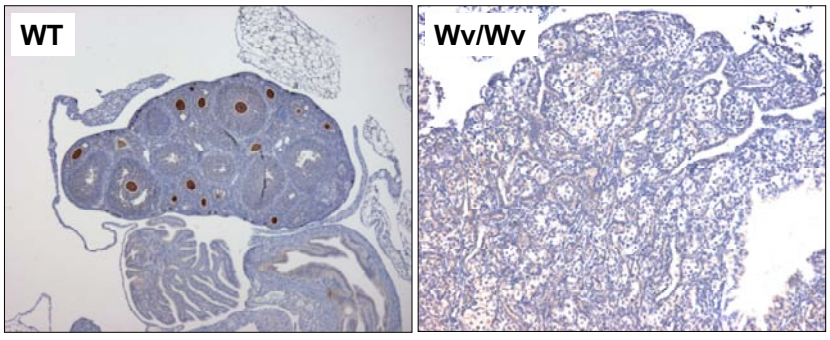
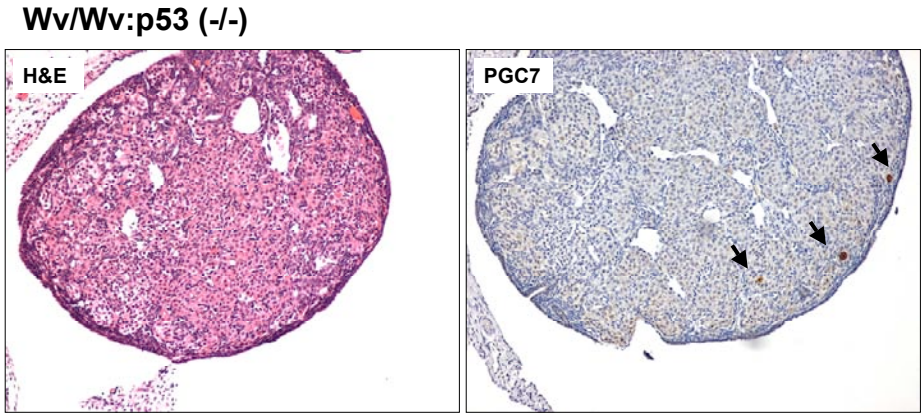


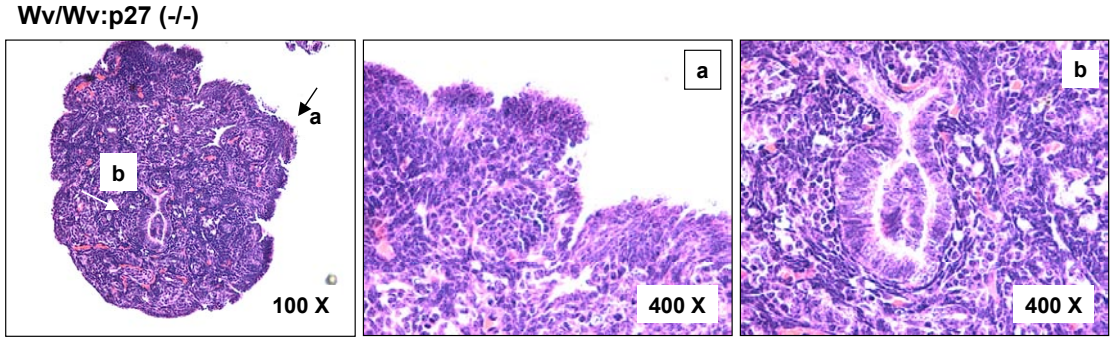
FIGURE 1. Staining of ovarian germ cells and follicles with PGC7 in wildtype and mutant mice: Representative immunostaining of follicles with germ cell marker PGC7 in ovaries from 3-month-old wildtype and Wv/Wv. The ovaries are representative of 5 mice analyzed in the pilot study of the **Aim 1**.

FIGURE 2. Ovarian morphology in Wv/Wv:p53 (-/-) ovaries. An H&E image and immunostaining with germ cell marker PGC7 of a representative ovary of the 3-month-old Wv/Wv:p53 (-/-) mice. H&E staining shows that there is no tumor lesion in the ovary. The presence of several germ cells or follicles are indicated by the positive staining of PGC7 (arrows) in a adjacent section of the same ovary. This ovary is representative of ovarian tissues harvested from 5 mice analyzed in the pilot study of the **Aim 1**.



The addition of p27 mutation into Wv/Wv mice altered the tumor phenotypes. The ovarian surface epithelia in these compound mutant mice, Wv/Wv:p27 (-/-) develop unique lesions with peculiar morphology, which are undergoing further analysis as planned in **Aim 2**. As shown in a representative ovary from a Wv/Wv:p27 (-/-) mouse, the ovarian tumor is smaller but shows morphology distinct from the Wv/Wv mice (**Figure 3**). We are investigating if these tumors are more malignant.

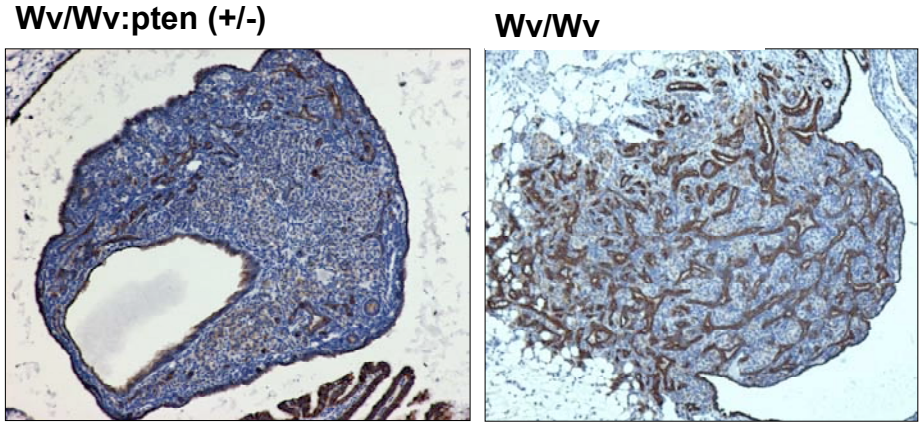
FIGURE 3. Ovarian morphology in Wv/Wv:p27 (-/-) ovaries. H&E images of a representative ovary of the 3-month-old Wv/Wv:p27 (-/-) mice. H&E staining shows the unique morphology of the ovarian tumor. The indicated areas (a, b, arrows) are shown in a higher magnification at left panels. This ovary is representative of ovarian tissues harvested from 5 mice analyzed in the pilot study of the **Aim 1**.



The addition of pten mutation into Wv/Wv mice reduced the presence of epithelial tumor lesions (**Figure 4**), but the ovaries from Wv/Wv:pten (+/-) often contain large cysts. Thus, we come to a conclusion that we need to introduce the additional mutations only to the surface epithelial cells but not to the germ cells of the Wv ovaries. The pilot study in **Aim 1.** (To

compare tumor phenotypes of Wv/Wv:p53 (-/-), Wv/Wv:p27 (-/-), and Wv/Wv:pten (+/-)) is now essentially completed. We chose to study the Wv/Wv:p53(flox/flox) model in details in *Task 2* (To characterize the selected ovarian tumor mouse model in detail (Months 13-36)). However, we consider to further investigate the ovarian tumors in Wv/Wv:p27 (-/-) mice since there are some interesting aspects of the tumor morphology. We will examine the ovarian tumors in older (12 months) Wv/Wv:p27 (-/-) mice to determine if these tumors will increase in size and malignancy, which will be part of the experimental work in *Task 2*.

FIGURE 4. Ovarian morphological features of Wv/Wv and Wv/Wv:pten (+/-) mutant mice: Representative cytokeratin staining (of epithelial cells) of ovaries from 3-month-old Wv/Wv and Wv/Wv:pten (+/-) mice. The figure shows the unique morphology and presence of a large cyst in the Wv/Wv:pten (+/-) ovary comparing to that od Wv/Wv. These ovaries are representative of ovarian tissues harvested from 5 mice each analyzed in the pilot study of the **Aim 1**.



In additional future study, we plan to create mutation only in ovarian surface epithelial cells to avoid the rescuing activity in germ cells by a cre-lox conditional gene targeting approach (**Aim 3**). Currently, we are crossing Wv mice into flox-p53 mutant mice. We will introduce cre recombinase into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells. This approach was considered in the original application as an alternative approach. Thus, *Task 2* has been initiated as planned. The future experiments will be carried out essentially as that were planned in the original application. The timeline and tasks in the Statement of Work have been well followed.

In the study of the Wv/Wv mice, we found/confirmed that Wv/Wv mice showed the depletion of germ cells/follicles and elevation of serum gonadotropins, mimicking peri-postmenopausal conditions in women. Thus, this study further suggests that the Wv/Wv mice are excellent model to study postmenipausal biology on ovarian cancer risk. These results have been included in a publication in press (Yang et al., Am. J. Pathology 2007).

To collaborate with the mouse model study, we have also examined human ovaries obtained from prophylactic oophorectomies for morphological changes as what we attempt to model using the Wv mice. We assembled a panel of archived ovarian tissues: 52 ovarian tissue blocks were from prophylactic oophorectomies of a high-risk (BRCA1/2 mutation or a family history of breast or ovarian cancer) population; 66 ovaries were from surgeries due to non-

ovarian-related diseases, referred to as normal-risk group. The morphology of ovarian tissues was examined, and morphological changes including papillomatosis, invaginations, inclusion cysts, and epithelial stratification were assessed in a blinded fashion. We found that inclusion cysts and deep invaginations were found much more commonly in women age 45–54 of either high risk or normal risk groups (**Figure 5**). When age was categorized into two groups, 45-54 representing peri-menopausal status and the other group consisting of the remaining age groups (below 45 years and 55 years & above), a statistically significant difference was found between age group and frequency of occurrence of morphological features. The odds of occurrence of inclusion cyst were 5.43 times as high in women aged 45-54 relative to other women (p-value = 0.009). Likewise, the odds of occurrence of deep invagination were 6.42 times as high in women aged 45-54 relative to other women (p-value = 0.008), and the odds of occurrence of Pseudo-stratification were 3.77 times as high in this group of women as in other women (p-value = 0.039). This study suggests that the frequency of these histological features, especially inclusion cysts, may associate with age or menopausal status. We propose that ovulatory and perimenopausal gonadotropin stimulation produces ovarian morphological changes, and these histological features may promote the transformation of genetically compromised epithelial cells in the development of ovarian cancer. This finding provides additional support for relevance and rationale to study the Wv mice as models to investigate menopausal physiology on ovarian epithelial remodeling and cancer risk.

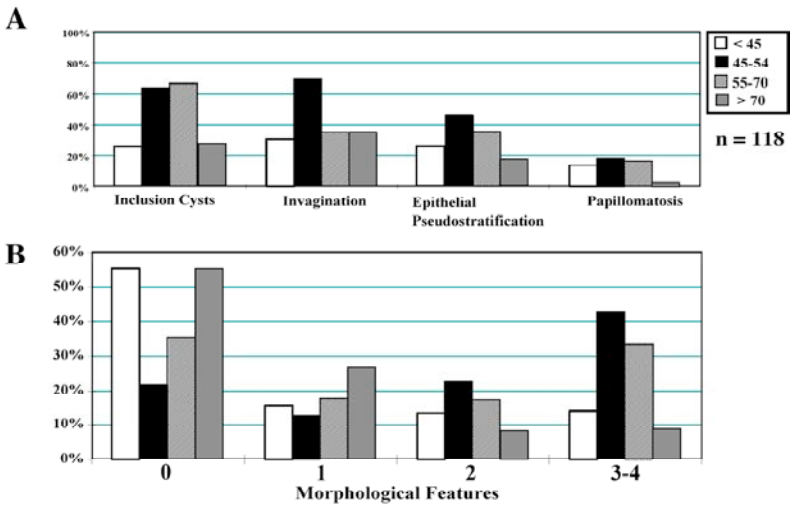


FIGURE 5. Ovarian morphological features: Correlation with age groups in all ovaries. All the 118 ovaries, including high risk and normal risk, are compiled and the morphological features are correlated with ages. (**A**) Morphological features are compared between each age group in the 118 ovaries. (**B**) The frequency of morphological features in an ovary is compared between age groups in the 118 ovaries. This data has been published in Cai et al., 2006 (**Appendices**).

Task 3 is "To analyze the alterations in signaling pathways in ovarian tumors and derived cell cultures from the mice (Months 30-36)". The work will be started in year 3, after the establishment and characterization of ovarian cancer mouse model. No experiments have been done in this task yet.

Therefore, we report that the project is progress well as planned in the first year. We found that the additional mutations had more impact on the germ cells than the epithelial cells. We have layered the basis in the first year of the project, and we hope to obtain conclusive results in the coming years.

3. Key Research Accomplishments

(1) Further verify the relevance of the Wv mouse model to human menopausal biology.

(2) Verifying the influence of reproductive aging/menopause on human ovarian morphological changes. This finding provides additional support for relevance and rationale to study the Wv mice as models to investigate menopausal physiology on ovarian epithelial remodeling and cancer risk.

(3) We made an unexpected but very interesting finding that the additional mutation of p53 in the Wv mice rescued ovarian tumor phenotype and preserved germ cells. This finding suggests p53 is very important for the survival and lifespan of ovarian germ cells and follicles. Also, the finding suggests that the depletion of follicles is key for the ovarian tumor phenotype.

4. Reportable Outcomes

(1) Yang et al., Am. J. Pathology, 2007 in press: We showed that the Wv mice are excellent models for human menopausal biology. This result is included in a paper in press.

(2) Cai et al., Gyn. Oncology 2006: We found that the human ovaries show age-dependent morphological changes, suggesting follicle depletion and gonadotropin stimulation in peri-menopausal period resulting in ovarian morphological changes. This observation has been published (Cai et al).

(3) Cai, Yang, Smith et al., 2007 in preparasion: We found that p53, pten, and p27kip1 genes have critical impacts on the survival of ovarian germ cells/follicle. We are doing additional experiments to characterize the mechanism further and also to obtain additional cases to determine the statistical significance. These results will be prepared for publication in 2-3 months.

5. Conclusions:

The experimental results are supportive of the hypothesis that. We need to delete p53 specifically in ovarian surface epithelial cells but not in germ cells tin order to create a model of malignant ovarian tumor based on the Wv mice.

In the future study, we plan to create mutation only in ovarian surface epithelial cells to avoid the rescuing activity in germ cells. Currently, we are crossing Wv mice into flox-p53 mutant mice. We will introduce cre recombinase into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells. This approach was considered in the original application as an alternative approach. Thus, the future experiments will be carried out essentially as that were planned in the original application.

In sum, the project is progress well as planned. We have layered the basis in the first year of the project, and we hope to obtain conclusive results for the aims and questions proposed in the coming years.

6. References

Cai, K.Q., Klein-Szanto, A., Karthik D., Edelson M., Daly M.B., Ozols R.F., Lynch H.T., Godwin A.K., **Xu X.X.** Age-dependent morphological alterations of human ovaries from populations with and without BRCA mutations. Gyn. Oncology, 2006 Nov;103(2):719-28.

Wan-Lin Yang, Andres Klein-Szanto, Thomas C. Hamilton and **Xiang-Xi Xu**. A reduction of Cox-2 gene dosage counters the menopausal ovarian morphological aging and tumour phenotype in Wv mice. 2006, resubmitted (Am. J. Pathology, in press, 2007).

7. Appendices

Cai, K.Q., Klein-Szanto, A., Karthik D., Edelson M., Daly M.B., Ozols R.F., Lynch H.T., Godwin A.K., **Xu X.X.** Age-dependent morphological alterations of human ovaries from populations with and without BRCA mutations. Gyn. Oncology, 2006 Nov;103(2):719-28.

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Age-dependent morphological alterations of human ovaries from populations with and without BRCA mutations ☆

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Abstract

Objective. From analysis of pre-cancer ovarian tissues obtained from prophylactic oophorectomies, several studies reported the increased ovarian morphological changes in high-risk ovaries, but whether these morphological changes are associated with BRCA1/BRCA2 genotypes or are cancer precursors is controversial. Here, we have investigated a recent collection of ovaries from prophylactic oophorectomies and control ovaries from surgeries due to other non-ovarian-related cancer or non-neoplastic diseases to determine if ovarian morphological changes relate to BRCA1/2 genotypes or reproductive history.

Methods. We assembled a panel of archived ovarian tissues: 52 ovarian tissue blocks were from prophylactic oophorectomies of a high-risk (HR) population; 66 ovaries were from surgeries due to non-ovarian-related diseases, referred to as normal-risk (NR) group. The morphology of ovarian tissues was examined, and morphological changes including papillomatosis, invaginations, inclusion cysts, and epithelial stratification were assessed in a blinded fashion.

Results. No statistically significant difference in frequency of these histolopathologic features was found between HR and NR groups. However, inclusion cysts and deep invaginations were found much more commonly in women age 45–54 of either HR or NR groups.

Conclusions. This study suggests that no significant increase in the presence of non-neoplastic ovarian morphological changes is associated with BRCA1/BRCA2 mutations. Rather, the frequency of these histological features, especially inclusion cysts, may associate with age or menopausal status. We propose that ovulatory and perimenopausal gonadotropin stimulation produces ovarian morphological changes, and these histological features may promote the transformation of genetically compromised epithelial cells in the development of ovarian cancer.

Keywords: Prophylactic oophorectomies; Aging; Menopause; Ovulation; Ovarian cancer; Epithelium; Pre-malignant lesion; Etiological factor; BRCA1/BRCA2

Introduction

The risk of epithelial ovarian cancer is known to associate with reproductive history, especially with ovulation. The same epidemiological data can be explained by two major theories, the incessant ovulation [1] and the gonadotropin stimulation hypotheses [2,3]. The incessant ovulation hypothesis postulates that the repetitive wounding and recurring cell proliferation in post-ovulatory repair of the ovarian surface epithelium result in mutations accumulating in the epithelial cells and ultimately tumor formation [4]. The gonadotropin theory postulates that the surges of pituitary gonadotropins that initiate each ovulation and persist in high levels for years following menopause also stimulate the ovarian surface epithelial cells and induce cell transformation. Abundant epidemiological data and animal models exist to support this idea [2–4]. Ovulation is an inflammatory-like process involving multiple cytokines and proteolytic enzymes, and their actions ultimately lead to tissue rupture [5,6]. Thus, inflammation of the ovarian epithelium was suggested as a mechanism by which ovulation contributes to

☆ The study finds that the dominant influence on ovarian morphological features is age and menopausal status, disregarding BRCA1 and BRCA2 genotypes. The finding may explain the contradictory reports on the association between ovarian morphology and BRCA genotype.

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cancer risk [7]. The pro-inflammatory cytokines are even higher in ovaries of perimenopausal stages [8]. Similarly, it is postulated that elevated gonadotropins in postmenopausal women may stimulate an inflammation-like reaction without leading to ovulation [9] and also may contribute to ovarian cancer risk, which is highest in peri-/postmenopausal stages [10].

A genetic component for ovarian cancer risk is evident, in that, other than age, a family history for ovarian cancer is the best prediction for ovarian cancer risk. Some of the predisposing mutations for the genetic factors are known, including germline mutations in *BRCA1* and *BRCA2* genes [11,12]. For women with identified family risk factors, prophylactic oophorectomy following the child-bearing period is considered a preventive approach for both breast and ovarian cancer [13].

Ovarian tissues from prophylactic surgeries of the high-risk population provide valuable materials for examination of preneoplastic lesions associated with genetic mutations or family history. One of the first studies reported the presence of benign

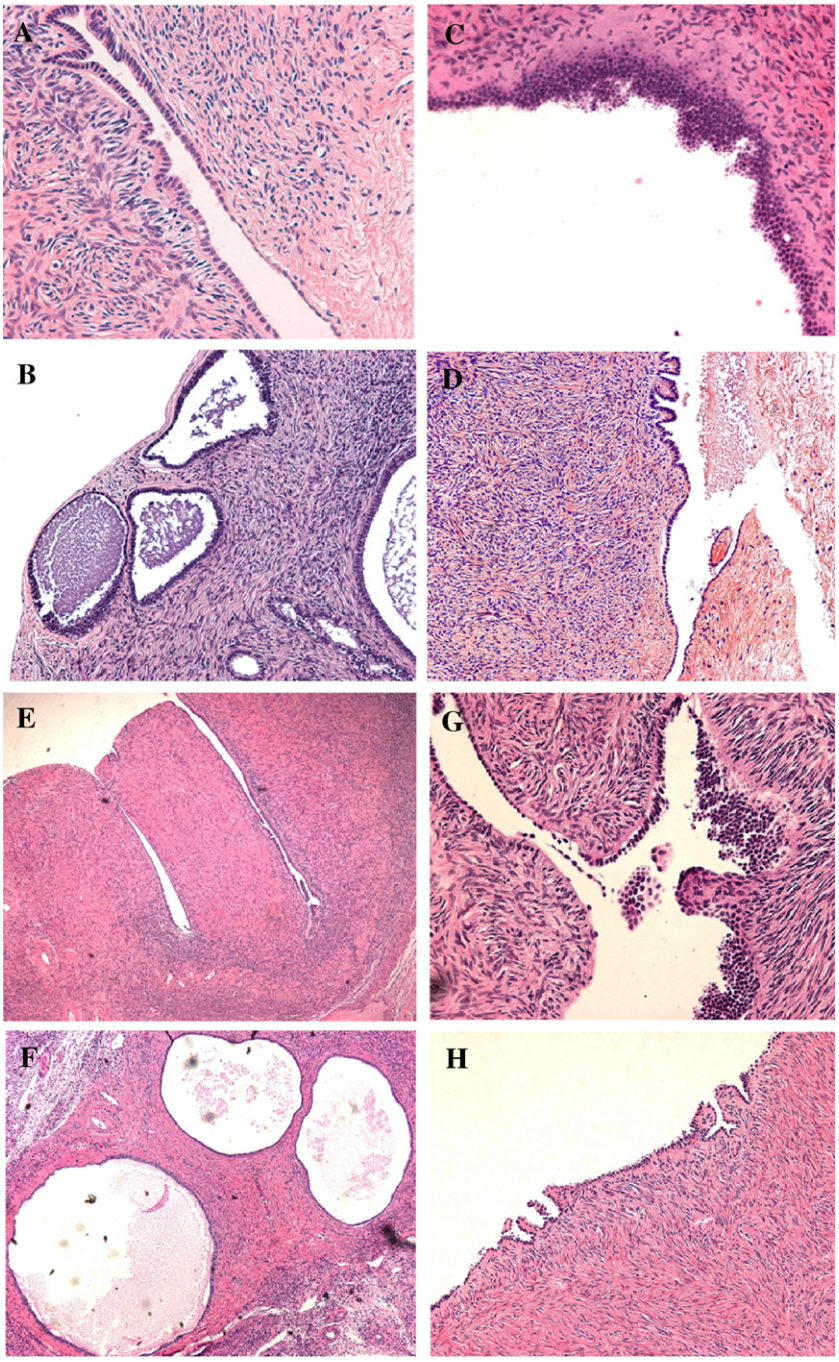

Fig. 1. Examples of ovarian morphological changes in prophylactic oophorectomies from high-risk individuals and from a normal-risk population. Typical examples of ovarian morphological changes from BRCA1/BRCA2 mutation carriers (A–D) or normal risk controls (E–H) are shown. (A) An example of surface deep invagination. (B) Presence of inclusion cysts. (C) Epithelial proliferation and pseudostratification. (D) Papillomatosis. (E) Surface deep invaginations. (F) Inclusion cysts. (G) Epithelial proliferation and pseudostratification. (H) Papillomatosis.

Table 1
Histologic changes in 52 prophylactic oophorectomies (HR group) and 66 controlled ovaries

Histological features	HR			NR
	BRCA1/2 (33 cases)	No mutation (19 cases)	Total (52 cases)	66 cases
Inclusion cyst	14 (42.4%)	10 (52.6%)	24 (46.2%)	35 (53.0%)
Deep invagination	17 (51.5%)	9 (47.4%)	26 (50.0%)	33 (50.0%)
Pseudostratification	14 (42.4%)	7 (36.8%)	21 (40.4%)	20 (30.3%)
Papillomatosis	9 (27.3%)	3 (15.8%)	12 (23.0%)	9 (13.6%)
0 feature	10 (30.3%)	7 (36.8%)	17 (32.7%)	23 (34.8%)
1 feature	7 (21.2%)	2 (10.5%)	9 (17.3%)	13 (19.7%)
2 features	7 (21.2%)	3 (15.8%)	10 (19.2%)	11 (16.7%)
3 or 4 features	10 (30.3%)	6 (31.6%)	16 (30.8%)	19 (28.8%)

Fifty-two archived ovarian tissue blocks from individual ovaries from prophylactic oophorectomies of a high-risk population were analyzed. The morphological features in the group classified as high-risk for ovarian cancer identified by either the presence of BRCA1/2 mutation (33 cases) or a family history were determined (19 cases). Sixty-six ovaries from surgeries from diseases unrelated to ovary were used as controls, the NR group. The cases containing morphological changes were identified, and percentage of the cases containing morphological changes was calculated. The number of features indicates the presence of a combination of morphological features including inclusion cyst, deep invagination, pseudostratification, or papillomatosis.

morphological changes and microscopic carcinomas in the BRCA1/BRCA2-associated ovaries [14]. Many subsequent studies (reviewed by Bell, 2005 [15]) reported the increased ovarian morphological changes in high-risk ovaries [16–19]. Others reported few histological changes but found morphological alterations of nuclei in BRCA1/BRCA2 mutant ovaries [20], and some studies demonstrated the presence of neoplastic phenotypes in surface epithelial cell cultures derived from the cancer-prone ovaries [21]. However, a significant number of studies found little difference between the ovaries of BRCA1/BRCA2 mutant carriers or normal risk controls [22–25]. Thus, whether BRCA1/BRCA2 genotypes lead to the ovarian pre-neoplastic morphological changes or if these morphological features are cancer precursors is not certain or generally agreed upon [15].

The observed morphological features, including papillomatosis, invaginations, inclusion cysts, and epithelial stratification/proliferation, may also be the result of ovarian "aging" [26,27]. Although no extensive data exist, the idea that ovarian morphological changes accumulate as the consequences of ovulation and post-ovulatory repair appears quite reasonable and is generally accepted [26,27].

To further explore the questions whether BRCA1/2 mutations lead to ovarian morphological changes and if these morphological alterations are the consequences of reproductive activity, we collected and examined a large panel of archived ovarian tissues from both prophylactic oophorectomies of a high-risk population and a population that is not associated with ovarian cancer, the normal-risk population. The presence and frequency of ovarian morphological features were analyzed to determine the potential correlation with BRCA mutant genotypes and reproductive stages (age).

Materials and methods

Patients and tissues

The case group, a set of 52 recently archived (years 2002–2004) human ovarian tissue specimens from prophylactic oophorectomies performed at Fox Chase Cancer Center and affiliated hospitals, was used. The majority of surgical oophorectomies were performed as a preventive approach in women with a genetic or perceived increased risk of developing ovarian cancer, i.e., *BRCA1* or *BRCA2* mutation carriers or reporting a personal and/or family history of disease. The criteria for prophylactic oophorectomy are the identification of a BRCA1 or BRCA2 mutation or a family history of predisposition to breast or ovarian cancer, specifically that 2 or more related family members had breast or ovarian cancer. The sequencing and genotyping of the BRCA1 and BRCA2 genes were done in the Clinical Molecular Genetic Laboratory at Fox Chase Cancer Center.

The control group consisted of normal ovarian tissues removed from 66 women for diseases unrelated to ovarian cancer. The control patients did not have a family history of ovarian or breast cancer, although a detailed questionnaire of health and family history is not available. In these patients, the diagnoses included endometrial adenocarcinoma in 35 cases, colorectal carcinoma in 8 cases, bladder cancer in 3 cases, lymphoma in 3 cases, and leiomyosarcoma in 2 cases. The remaining 15 cases were from patients who had benign diseases not involving the ovary. After excluding the ovaries of the 35 cases from endometrial carcinomas, 31 cases remained and were used as a subgroup for further control.

Histories of family cancer and identified germline mutations were obtained without reference to the patients' personal information. The use of these human tissues in our research has been examined and approved by the Institutional Review Board Committee (IRB), and safety and ethical guidelines were followed in using the human tumor tissues according to institutional requirements. To preserve privacy, a series of security procedures were undertaken. Each study subject was given a unique numeric identifier upon study entry. Biological specimens were only labeled with this unique biosample identifier. Neither the individual's name nor ID appeared on the biosample. Furthermore, all personnel

Table 2
Correlation between histopathologic changes and age in 52 prophylactic oophorectomies (the HR group)

Histological features	Age group			
	<45 (28 cases)	45–54 (18 cases)	55–70 (4 cases)	>70 (2 cases)
Inclusion cyst	7 (25%)	13 (72.2%)	4 (100%)	0 (0%)
Deep invagination	9 (32.1%)	14 (77.8%)	2 (50%)	1 (50%)
Pseudostratification	8 (28.6%)	11 (61.1%)	2 (50%)	0 (0%)
Papillomatosis	5 (17.9%)	6 (33.3%)	1 (25%)	0 (0%)
0 feature	15 (53.6%)	1 (5.6%)	0 (0%)	1 (50%)
1 feature	4 (14.3%)	3 (16.7%)	1 (25%)	1 (50%)
2 features	5 (17.9%)	4 (22.2%)	1 (25%)	0 (0%)
3 or 4 features	4 (14.3%)	10 (55.6%)	2 (50%)	0 (0%)

The morphological features in the HR group were determined and presented in 4 age groups.

Table 3
Correlation between histopathologic changes and patient age in 66 non-ovarian cancer control group (NR)

Histological features	Age group			
	<45 (8 cases)	45–54 (29 cases)	55–70 (20 cases)	>70 (9 cases)
Inclusion cyst	2 (25%)	16 (55.2%)	14 (70%)	3 (33.3%)
Deep invagination	2 (25%)	18 (62.1%)	10 (50%)	3 (33.3%)
Pseudostratification	1 (12.5%)	11 (37.9%)	7 (35%)	1 (11.1%)
Papillomatosis	1 (12.5%)	3 (10.3%)	5 (25%)	0 (0%)
0 feature	5 (62.5%)	9 (31.0%)	4 (20%)	5 (55.6%)
1 feature	2 (25%)	3 (10.3%)	6 (30%)	2 (22.2%)
2 features	0 (0%)	7 (24.1%)	3 (15%)	1 (11.1%)
3 or 4 features	1 (12.5%)	10 (34.5%)	7 (35%)	1 (11.1%)

Table 4
Correlation between histopathologic changes and age in all ovaries (52 RH plus 66 NR)

Histological features	Age group				Total (118 cases)
	<45 (36 cases)	45–54 (47 cases)	55–70 (24 cases)	>70 (11 cases)	
Inclusion cyst	9 (25%)	29 (61.7%)	18 (63.6%)	3 (27.3%)	59 (50.0%)
Deep invagination	11 (30.5%)	32 (68.1%)	12 (36.4%)	4 (36.4%)	59 (50.0%)
Pseudostratification	9 (25%)	22 (46.8%)	9 (36.4%)	1 (9.1%)	41 (34.7%)
Papillomatosis	6 (16.7%)	9 (19.1%)	6 (18.2%)	0 (0%)	21 (17.8%)
0 feature	20 (55.6%)	10 (21.3%)	4 (36.4%)	6 (54.5%)	40 (33.9%)
1 feature	6 (16.7%)	6 (12.8%)	7 (18.2%)	3 (27.3%)	22 (18.6%)
2 features	5 (13.9%)	11 (23.4%)	4 (16.7%)	1 (9.1%)	21 (18.6%)
3 or 4 features	5 (13.9%)	20 (42.6%)	8 (33.3%)	1 (9.1%)	35 (29.7%)

The morphological features of the total ovaries ($n=118$, combining HR and NR groups) are summarized and presented in 4 age groups.

associated with this study have received HIPAA and human subjects protection training and have been certified by the IRB. As a result, the study is retrospective, and we did not have the ability to add additional components or examine additional factors that were not planned initially.

Morphologic evaluation (histopathology)

Five-micron sections of the ovaries (one to four slides per case) were stained with hematoxylin–eosin and examined by a pathologist blinded to the case or control designation of the tissues. Morphologic changes scored included epithelial inclusion cysts, epithelial deep invaginations, epithelial pseudostratification, and papillomatosis.

In most of the cases, one archived ovary per individual is available, and the other ovary from the donor was used for preparation of mRNA or ovarian surface epithelial cells in other studies. The ovarian tissues from the institutional tumor bank are used by multiple investigators. and we were not able to section and sample through an entire ovary in our analysis. In 5 cases that we were able to section through the whole tissue block, no microscopic tumors or unusual features were found. In the quantification of ovarian lesions, we only used one section (about 2 × 1 cm^2) per ovary. In those cases where several tissue blocks from a single ovary were available, we chose information from the largest block for analysis and calculation.

Immunohistochemistry

The tissue sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Then, the sections were subjected to antigen retrieval by boiling in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven for 10 min. After blocking endogenous peroxidase activity by using 0.3% hydrogen peroxide and blocking in 1.5% normal goat serum, the sections were incubated overnight with mouse monoclonal anti-p53 (1:300), anti-Ki67 (1:150), or anti-CA125 (1:500) at 4°C in a humidified chamber. Biotinylated goat anti-mouse IgG antibodies were incubated with the sections for 30 min at room temperature. The antibody complex was detected by the LSAB system (Dako) and was visualized with the chromogen 3,3′-diaminobenzidine. Sections were lightly counterstained with hematoxylin. The primary antibody was replaced with 1× PBS as a negative control.

Statistical analysis

Statistical analysis was performed using two-sided Fisher's Exact Test at the 5% significance level (software R version 2.1.0.) to compare morphological changes with mutation status and patient age. All relevant P values and odds ratios were calculated and reported by a statistician (K Devarajan).

Results

Morphological features of ovarian tissues

We assembled a recent collection of prophylactic oophorectomies and control ovaries at the Fox Chase Cancer Center Tumor Bank for analysis. The tissues obtained for this study included 52 ovarian tissue blocks from prophylactic oophorectomies of a high-risk (HR) population either by the criteria of identified BRCA1/BRCA2 mutations or by family history. In this HR group, 19 cases were identified as BRCA1 mutation carriers, 13 as BRCA2 mutation carriers, 1 case contained both BRCA1 and BRCA2 mutations, and 19 cases were negative for either BRCA1 and BRCA2 mutations but the patients opted for prophylactic oophorectomies based on a high-risk family history. The control, normal-risk (NR) group consisted of 66 ovaries obtained from surgeries due to non-ovarian diseases. In these patients, the diagnoses included 35 endometrial adenocarcinomas, 8 colorectal carcinomas, 3 bladder cancer cases, 3 lymphomas, 2 leiomyosarcoma, and 15 cases of benign diseases not involving the ovary.

One H&E-stained section from each ovarian tissue, of approximately 1 × 2 cm in size, was surveyed for morphological features by a pathologist blinded to the case or control designation of the tissues. In this set of tissues, either from HR or NR, no neoplastic lesions were found. Few follicles were present in the tissues. General morphological changes, including epithelial inclusion cysts, epithelial deep invaginations, epithelial proliferation/pseudostratification, and papillomatosis, were noted in both high- and normal risk-populations (Fig. 1). The general features of the ovaries are scored and listed in Tables 1–4.

Histological characterization of morphological lesions

Although the Fox Chase Cancer Center group has identified microscopic carcinomas in prophylactic oophorectomies previously [14] and the presence of microscopic tumors in prophylactic oophorectomies was also found subsequently by several other groups [18,19], we did not find overt cancer or microscopic malignancies in this collection of ovaries. Of note, however, are two benign lesions, a cystadenoma and a papillary cystadenoma, identified in the ovary of a BRCA2 mutation carrier (Figs. 2A, B). Mild epithelial atypia was occasionally observed in prophylactic oophorectomies, as shown by two examples (Figs. 2C–F and G–J). In a typical ovarian inclusion cyst (Fig. 2C), the epithelial cells lining the cyst surface appeared to demonstrate mild atypia (Fig. 2D). The cyst epithelial cells were also positive for CA125 staining on the apical surface (Fig. 2E), and the cells stained strongly positive

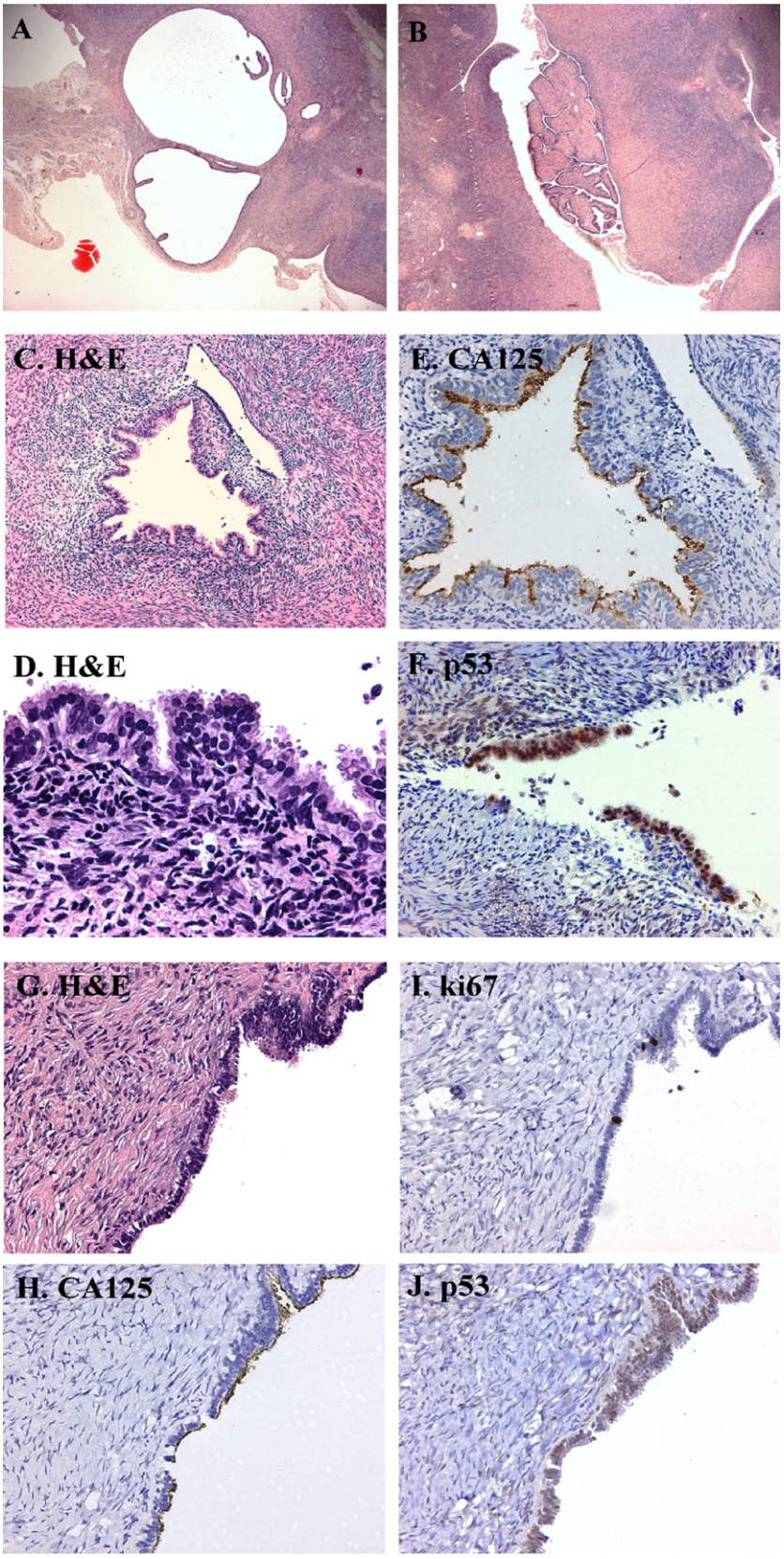

Fig. 2. Pre-neoplastic morphological phenotypes and molecular alterations in prophylactic oophorectomies. Ovarian tissues from prophylactic oophorectomies were subjected to histological analysis, and representative examples of lesions and the characterization by immunohistochemistry are shown. (A) Cystadenoma. (B) Papillary adenofibroma. (C–F) Characterization of an ovarian inclusion cyst: (C) H&E. (D) Mild epithelial atypia is seen in a higher magnification. (E) CA125 staining is positive in the epithelial cells lining the larger cyst. (F) The epithelial cells are strongly positive for p53 staining. (G–J) Characterization of an ovarian surface epithelial lesion. (G) Mild atypia is observed in ovarian surface epithelial cells, H&E staining. (H) The epithelial cells are positive for CA125. (I) Ki67-positive cells are present. (J) The epithelial cells are weakly p53 positive.

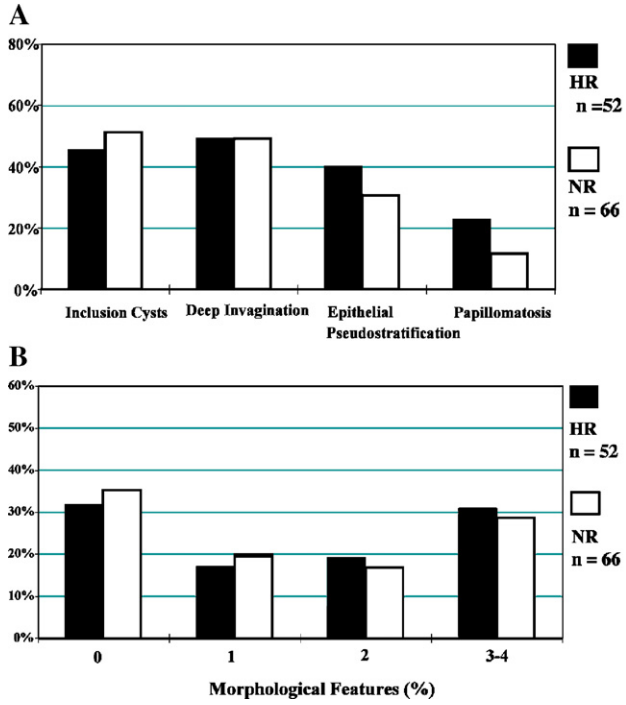

Fig. 3. Ovarian morphological features: no significant difference between high- and normal-risk groups. Fifty-two ovarian tissues from prophylactic oophorectomies of a high-risk population for breast and ovarian cancer and 66 ovaries from surgeries of non-ovarian-related diseases (classified as normal risk) were collected. One section from each ovarian tissue sample with H&E staining was examined in a blinded manner for morphological features and the numbers of features present in an ovary. The frequency for the presence of morphological features was compared. (A) The frequencies of each morphological feature including inclusion cysts, invagination, pseudostratification, and papillomatosis are compared between HR and NR groups. (B) The numbers of morphological features are compared between HR and NR groups.

for nuclear p53 (Fig. 2F). In one example of ovarian surface epithelial cells, mild atypia was also seen (Fig. 2G). The cells were also positive for CA125 (Fig. 2H) and weakly positive for p53 (Fig. 2J). Ki67-positive cells were present, indicating active epithelial proliferation (Fig. 2I). In the cases other than atypia, no remarkable staining for p53, Ki67, and CA125 was observed. Thus, the epithelial morphological lesions are associated with molecular alterations. However, epithelial atypia is not frequent: we observed 3 cases in ovaries from the HR population, and none was observed in the NR ovaries.

Comparison of histological features between ovaries from high- and normal-risk populations

Following morphological analysis of all ovarian tissues, we summarized the results to compare the morphological features seen in each group. Within the HR group, the 52 ovaries were divided into two subgroups: the group identified to be BRCA1/BRCA2 mutation carriers ($n = 33$) and the group identified to have a family history of ovarian cancer but with no identified BRCA1/BRCA2 mutations ($n = 19$). The only noted difference is the percentage of papillomatosis, 9/33 (27.3%) in BRCA1/BRCA2 mutation carriers and 3/19 (15.8%) in the non-BRCA1/BRCA2 group (Table 1). However, this difference was found to have no sufficient statistical significance (P value = 0.4985 using the two-sided Fisher's Exact Test). The frequency of the accumulation of morphological features is also very similar in both subgroups. Thus, basically, we found no obvious difference in morphological features between these two subgroups (Table 1).

Within the NR group, we compared the morphological features between the 35 cases of endometrium adenocarcinomas and the other 31 ovaries. No notable difference was seen, suggesting that the ovaries were not affected by the presence of nearby endometrial cancer. Thus, we used all 66 ovaries as the control group for further analysis.

The morphological features of the 52 ovaries in the HR group were then compared to these 66 in NR group (Table 1, Fig. 3). There was no difference in the frequency of the presence of inclusion cysts and invaginations between the HR and NR groups; however, it appears that the HR group may have slightly more epithelial pseudostratification and papillomatosis than the NR group (Fig. 3A), comparing 21/52 (40.4%) to 20/66 (30.3%) for the frequency of epithelial pseudostratification, and 12/52 (23.0%) to 9/66 (13.6%) for the frequency of papillomatosis, of HR to NR, respectively. Nevertheless, the likelihood of an ovary to have more than one morphological feature is similar between HR and NR groups (Table 1, Fig. 3A). Furthermore, no statistically significant difference was found from these two sets of data in the analysis for association between HR/NR status and the frequency of each of the histological features by Fisher's Exact Test.

Age-dependent ovarian morphological changes

Although a BRCA1/BRCA2 genotype–morphological relationship was not found, we observed age-dependent ovarian morphological changes in all groups (Fig. 4). We divided the patients into 4 different age groups: <45 years old for premenopausal women, 45–54 years of age for perimenopausal women, 55–70 years for postmenopausal women, and >70 years for aged ovaries. This age stratification also ensured that each age group contained a comparable number of samples for representation. In both the HR and NR groups, the occurrence of morphological features of inclusion cysts, epithelial inclusion cysts, epithelial deep invaginations, epithelial pseudostratification, and papillomatosis distributes along a bell-shape curve depending on age (Fig. 4). Again, parallel patterns can be seen comparing the age-dependent morphological features between the HR and the NR groups (Fig. 4). For example, the presence of inclusion cysts is low (25%) in premenopausal women, increased in perimenopausal women (55–73%), and highest (70–100%) in postmenopausal women. However, the presence of inclusion cysts is decreased in aged ovaries (0–33%). Invaginations were observed most frequently in perimenopausal women in both HR and NR groups. Both epithelial pseudostratification and papillomatosis exhibit age-dependent changes but are not as dramatic. For ovaries with multiple (three to four) morphological features (Figs. 4C, D), the frequency is highest in peri- and postmenopausal women, but lower in younger and aged ovaries.

Since the difference between the HR and the NR groups is quite subtle but the age-dependent morphological features

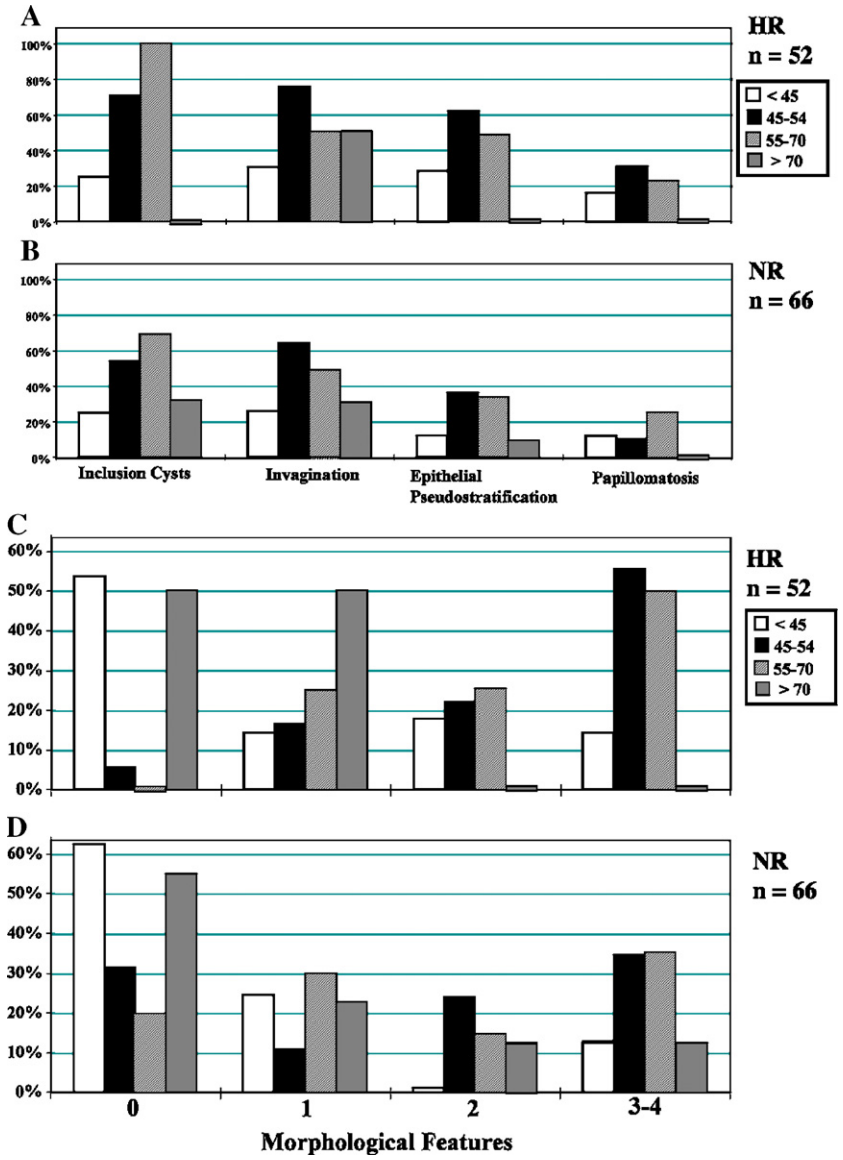

Fig. 4. Ovarian morphological features: correlation with age groups in either HR or NR ovaries. The ovarian donors were divided into 4 age groups: <45, 45–54, 55–70, and >70. (A) Each morphological feature is compared between age groups in the 52 HR ovaries. (B) Each morphological feature is compared between age groups in the 66 NR ovaries. (C) The number of morphological features in an ovary is compared between age groups in the 52 HR ovaries. (D) The number of morphological features in an ovary is compared between age groups in the 66 NR ovaries.

are obvious in both groups, we combined all ovaries and determined the morphological features in each age group with a larger number (n = 118) of samples (Fig. 5). The age-dependent morphological alteration is obvious: perimenopausal ovaries (45–55 years) exhibit inclusion cysts and epithelial deep invagination most frequently (Fig. 5A); postmenopausal ovaries (56–70 years) also show frequent invaginations (Fig. 5A); and both younger and older ovaries have fewer epithelial morphological changes. Furthermore, ovaries from women between the ages 45 and 70 have the most morphological features (Fig. 5B). Thus, we observed a correlation of ovarian morphological changes with ages in the current analysis.

We also used mathematical model to perform two-sided comparison for association between age and morphological features in the pooled data. When we divided the age into two groups, younger or older than age 50, the association between age and morphological features is not statistically significant. However, when age was categorized into two groups, 45–54 representing perimenopausal status and the other group consisting of the remaining age groups (below 45 years and 55 years and above), a statistically significant difference was found between age group and frequency of occurrence of morphological features. The odds of occurrence of inclusion cyst were 5.43 times as high in women aged 45–54 relative to other women (P value = 0.009). Likewise, the odds of occurrence of deep invagination were 6.42 times as high in women aged 45–54 relative to other women (P value = 0.008), and the odds of occurrence of pseudostratification were 3.77 times as high in this group of women as in other

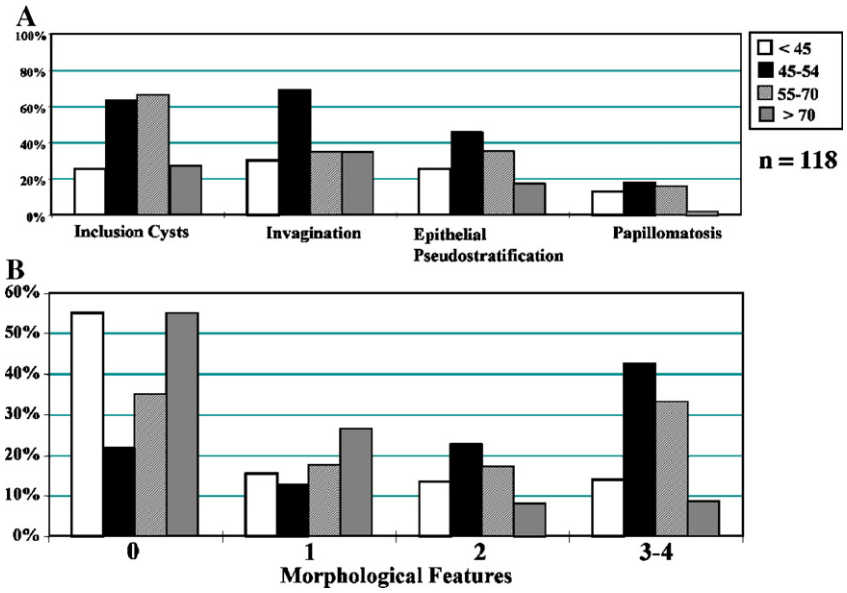

Fig. 5. Ovarian morphological features: correlation with age groups in all ovaries. All the 118 ovaries, including HR and NR, are compiled, and the morphological features are correlated with ages. (A) Morphological features are compared between each age group in the 118 ovaries. (B) The frequency of morphological features in an ovary is compared between age groups in the 118 ovaries.

women (P value = 0.039). Thus, the association between age and ovarian morphological feature is not simply younger and older but is related to perimenopausal status.

Discussion

The presence of pre-neoplastic lesions in ovaries from prophylactic oophorectomies, determined either by morphological or molecular criteria, has been investigated by many groups, with discrepancies in the results and conclusions [15]. The Fox Chase Cancer Center group first reported the finding of benign to malignant microscopic tumors in prophylactic oophorectomies [14]. The presence of microscopic tumors in prophylactic ovaries has been confirmed by many groups [18,19]. We did not find overt microscopic malignancy in the current collection of ovaries. This is a potential caveat that this set of ovaries may be pre-selected, that ovaries containing neoplasm have been used for diagnostic purpose and were not distributed to the tumor bank. Additionally, we have not sectioned through the entire ovary of each sample and thus potentially failed to find the existing microscopic tumors.

The current study of ovaries from prophylactic oophorectomies is unique in the analysis and finding of morphological changes in relation to age and menopausal status. The current study did find some potential differences between the HR group and the NR group in ovarian morphological features, especially in epithelial pseudostratification and papillomatosis. However, the dominant influence on ovarian morphological features is age and menopausal status of the ovaries, disregarding the genotypes or risk status of the ovaries. We concluded that perimenopausal (age 45–55) and postmenopausal (age 56–70) ovaries exhibit most morphological changes. Younger (less than 45 years old) and older (more than 70 years old) ovaries have the least morphological changes.

Thus, BRCA1/BRCA2 mutant genotype may not be associated with non-malignant morphological alterations, a finding consistent with previous studies [22–25]. Nevertheless, genetic mutations may associate with the increase incident of atypia and microscopic tumors observed previously [14,18,19]. The lack of significant number of atypia and microscopic tumors in the current set of ovarian tissues forbids us to examine this point.

The idea of age-dependent ovarian morphological changes, so-called “ovarian aging”, was proposed previously from pathologic observation that ovaries from older women exhibit more morphological changes, and it was suggested that such morphological changes are caused by ovulation and subsequent repair [26,27]. Our analysis provides quantitative data to support the general idea of ovarian morphological aging in humans.

The result suggests that, in the peri- and postmenopausal periods, the ovarian morphological changes are high. The phenomenon of menopause is a unique feature of human females and a result of an extension of human lifespan due to the recent advances in medicine [28,29]. Menopause, the post-reproductive period, is caused by depletion of ovarian follicles. Following cessation of ovulation, an endocrine feedback loop is diminished and the production of pituitary gonadotropins is elevated [28–30]. An elevated gonadotropin level in peri- and postmenopausal period is likely the causative factor for ovarian morphological “aging”. Although the high gonadotropins no longer initiate ovulation, the hormones may stimulate inflammatory reactions mimicking ovulation in the epithelial compartment and promoting epithelial morphological change [9,31]. That this menopausal increase in gonadotropins is a causative factor for ovarian morphological changes is supported by the observation of the germ-cell-deficient W mouse model [32]. Once reproductive age is reached, ovarian follicles are

rapidly depleted, and gonadotropins are elevated in these mice [33]. Subsequently, the ovaries of W mice acquire pronounced epithelial morphological changes, forming surface invagination, inclusion cysts, papillomatosis, and develop benign ovarian tumors [34], which can be rescued by antagonists to gonadotropins [35].

In older women (age 70 or older), the gonadotropin level is reduced and the ovaries are also reduced in size [36]. Tissue remodeling may resolve the morphological features that developed during the menopausal period and explain our observation of a reduction in ovarian morphological features in the older age group.

Thus, our observation leads us to speculate that the menopausal elevation of gonadotropin level is the predominant factor (rather than BRCA1/BRCA2 genotypes) in determining the morphological features and their age-dependent changes in ovaries.

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References

[1] Fathalla MF. Incessant ovulation—A factor in ovarian neoplasia? Lancet 1971;2:163.
[2] Cramer DW, Welch WR. Determinants of ovarian cancer risk: II. Inferences regarding pathogenesis. J Natl Cancer Inst 1983;71:717–21.
[3] Mohle J, Whittemore A, Pike M, Darby S. Gonadotrophins and ovarian cancer risk. J Natl Cancer Inst 1985;75:178–80.
[4] Riman T, Persson I, Nilsson S. Hormonal aspects of epithelial ovarian cancer: review of epidemiological evidence. Clin Endocrinol (Oxf) 1998;49:695–707.
[5] Richards JS, Russell DL, Ochsner S, Espey LL. Ovulation: new dimensions and new regulators of the inflammatory-like response. Annu Rev Physiol 2002;64:69–92.
[6] Tsafriri A, Reich R. Molecular aspects of mammalian ovulation. Exp Clin Endocrinol Diabetes 1999;107:1–11.
[7] Ness RB, Cottreau C. Possible role of ovarian epithelial inflammation in ovarian cancer. J Natl Cancer Inst 1999;91:1459–67 [RESPONSE: J Natl Cancer Inst. 2000; 92:163].
[8] Pfeilschifter J, Koditz R, Pfohl M, Schatz H. Changes in proinflammatory cytokine activity after menopause. Endocr Rev 2002;23:90–119.
[9] Smith ER, Xu XX. Etiology of epithelial ovarian cancer: a cellular mechanism for the role of gonadotropins. Gynecol Oncol 2003;91:1–2.
[10] Tortolero-Luna G, Mitchell MF. The epidemiology of ovarian cancer. J Cell Biochem Suppl 1995;23:200–7.
[11] Boyd J, Rubin SC. Hereditary ovarian cancer: molecular genetics and clinical implications. Gynecol Oncol 1997;64:196–206.
[12] Rubin SC, Benjamin I, Behbakht K, Takahashi H, Morgan MA, LiVolsi VA, Berchuck A, Muto MG, Garber JE, Weber BL, Lynch HT, Boyd J. Clinical and pathological features of ovarian cancer in women with germ-line mutations of BRCA1. N Engl J Med 1996; 335:1413–6.
[13] Haber D. Prophylactic oophorectomy to reduce the risk of ovarian and breast cancer in carriers of BRCA mutations. N Engl J Med 2002; 346:1660–2.
[14] Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, et al. Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. J Natl Cancer Inst 1996;88:1810–20.
[15] Bell DA. Origins and molecular pathology of ovarian cancer. Mod Pathol 2005;18(Suppl 2):S19–32.
[16] Deligdisch L, Gil J, Kerner H, Wu HS, Beck D, Gershoni-Baruch R. Ovarian dysplasia in prophylactic oophorectomy specimens: cytogenetic and morphometric correlations. Cancer 1999;86:1544–50.
[17] Schlosshauer PW, Cohen CJ, Penault-Llorca F, Miranda CR, Bignon YJ, Dauplat J, Deligdisch L. Prophylactic oophorectomy: a morphologic and immunohistochemical study. Cancer 2003;98:2599–606.
[18] Colgan TJ, Murphy J, Cole DE, Narod S, Rosen B. Occult carcinoma in prophylactic oophorectomy specimens: prevalence and association with BRCA germline mutation status. Am J Surg Pathol 2001;25:1283–9.
[19] Lu KH, Garber JE, Cramer DW, Welch WR, Niloff J, Schrag D, Berkowitz RS, Muto MG. Occult ovarian tumors in women with BRCA1 or BRCA2 mutations undergoing prophylactic oophorectomy. J Clin Oncol 2000;18: 2728–32.
[20] Werness BA, Afify AM, Bielat KL, Eltabbakh GH, Piver MS, Paterson JM. Altered surface and cyst epithelium of ovaries removed prophylactically from women with a family history of ovarian cancer. Hum Pathol 1999;30:151–7.
[21] Dyck HG, Hamilton TC, Godwin AK, Lynch HT, Maines-Bandiera S, Auersperg N. Autonomy of the epithelial phenotype in human ovarian surface epithelium: changes with neoplastic progression and with a family history of ovarian cancer. Int J Cancer 1996;69:429–36.
[22] Barakat RR, Federici MG, Saigo PE, Robson ME, Offit K, Boyd J. Absence of premalignant histologic, molecular, or cell biologic alterations in prophylactic oophorectomy specimens from BRCA1 heterozygotes. Cancer 2000;89:383–90.
[23] Leeper K, Garcia R, Swisher E, Goff B, Greer B, Paley P. Pathologic findings in prophylactic oophorectomy specimens in high-risk women. Gynecol Oncol 2002;87:52–6.
[24] Stratton JF, Buckley CH, Lowe D, Ponder BA. Comparison of prophylactic oophorectomy specimens from carriers and noncarriers of a BRCA1 or BRCA2 gene mutation. United Kingdom Coordinating Committee on Cancer Research (UKCCCR) Familial Ovarian Cancer Study Group. J Natl Cancer Inst 1999;91:626–8.
[25] Casey MJ, Bewtra C, Hoehne LL, Tatpati AD, Lynch HT, Watson P. Histology of prophylactically removed ovaries from BRCA1 and BRCA2 mutation carriers compared with noncarriers in hereditary breast ovarian cancer syndrome kindreds. Gynecol Oncol 2000;78:278–87.
[26] Nicosia SV. The aging ovary. Med Clin North Am 1987;71:1–9.
[27] Finn CA. Reproductive ageing and the menopause. Int J Dev Biol 2001;45: 613–7.
[28] Lobo RA, Kelsey J, Marcus R, editors. Menopause: biology and pathobiology. 1st ed. Academic Press; 2000.
[29] Gosden RG, editor. Biology of menopause: the causes and consequences of ovarian aging. Academic Press; 1985.
[30] Eldridge JC, McPherson III JC, Mahesh VB. Maturation of the negative feedback control of gonadotropin secretion in the female rat. Endocrinology 1974;94:1536–40.
[31] Yang WL, Godwin AK, Xu XX. Tumor necrosis factor-alpha-induced matrix proteolytic enzyme production and basement membrane remodeling by human ovarian surface epithelial cells: molecular basis linking ovulation and cancer risk. Cancer Res 2004;64:1534–40.
[32] Murphy ED. Hyperplastic and early neoplastic changes in the ovaries of mice after genic deletion of germ cells. J Natl Cancer Inst 1972;48: 1283–95.

[33] Murphy ED, Beamer WG. Plasma gonadotropin levels during early stages of ovarian tumorigenesis in mice of the Wx–Wv genotype. Cancer Res 1973;33:721–3.
[34] Yang, WL, Klein-Szanto, A, Hamilton, TC, Xu, XX, A reduction of Cox-2 gene dosage counters the menopausal ovarian morphological aging and tumour phenotype in Wv mice. submitted for publication.
[35] Blaakaer J, Baeksted M, Micic S. Gonadotropin-releasing hormone agonist suppression of ovarian tumorigenesis in mice of the Wx/Wv genotype. Biol Reprod 1995;53:775–9.
[36] Pavlik EJ, Liu C, DePriest PD, Gallion HH, Ueland FR, Kryscio RJ, van Nagell Jr JR. Relating ovarian size to age, menopausal status, and use of hormones. Gynecol Oncol 2001;80:333–4.

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AQ: A

A Reduction of Cyclooxygenase 2 Gene Dosage Counters the Ovarian Morphological Aging and Tumor Phenotype in Wv Mice

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Menopausal ovaries undergo morphological changes, known as ovarian aging, which are implicated in the high incidence of ovarian cancer occurring during the perimenopausal and immediate postmenopausal periods. The germ cell-deficient Wv mice recapitulate these postmenopausal alterations in ovarian morphology and develop tubular adenomas. We demonstrate that a reduction of cyclooxygenase 2 gene dosage rescued the ovarian aging phenotype of the Wv mice, whereas homozygous deletion was accompanied by a compensatory increase in ovarian cyclooxygenase 1 expression and prostaglandin E_2 synthesis. Cyclooxygenase inhibitors also reduced the tumor phenotype in a preliminary study. These findings suggest that increased cyclooxygenase activity contributes to the preneoplastic morphological changes of the ovarian surface epithelium, which can be reversed by a reduction of gene dosage achieved by either genetic or pharmacological approaches. ***(Am J Pathol 2007, 170:000–000; DOI: 10.2353/ajpath.2007.060769)***

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Menopause is defined as the permanent cessation of menstruation resulting from depletion of germ cells and loss of ovarian follicular activity, and it is accepted to be a by-product of modern health advances and the extension of lifespan that occurred in the last century.[1] By the end of the reproductive age, germ cells and follicles are depleted from the ovaries, and the ovulatory cycle ceases, resulting in menopause. The perimenopausal period commences when the first features of menopause begin until at least 1 year after the final menstrual period, generally lasting an average of 5 years. In humans, the transition to menopause is a set of gradual changes, in which ovarian function, reproductive capacity, and hormonal status are altered long before menses stops completely. Menopause generally occurs between 45 to 55 years of age, and the symptoms vary among women.

After menopause, estrogen levels fall, but the gonadotropins including luteinizing hormone and follicle-stimulating hormone (FSH) are elevated and often even higher than before menopause.[1] The incidence of ovarian cancer is highest in the perimenopausal period, which supports the gonadotropin stimulation theory of ovarian cancer etiology. Among the physiological changes associated with menopause, the ovarian tissues undergo morphological transformation, known as ovarian aging, and this is implicated in the high incidence of ovarian cancer that occurs during the perimenopausal and immediate postmenopausal periods.[1–4] One feature associated with ovarian aging is the accumulation of ovarian morphological changes such as deep invaginations, surface papillomatosis, and inclusion cysts (Supplementary Figure 1, see *http://ajp.amjpathol.org*), which are thought by some to be the histological precursors of ovarian cancer.[3,4]

The phenomenon of menopause is not restricted to human females but also occurs in laboratory rats and mice that exhibit postreproductive lifespan preceded by a period of gradual reproductive decline. A naturally occurring mutant, white spotting variant (Wv) mice harbor a point mutation in the kinase domain of the c-kit gene, resulting in developmental defects in germ cells, pigment-forming cells, red blood cells, and mast cells in homozygous mutant mice.[5–8] The Wv/Wv mice have a similar lifespan as wild type, are sterile, white coated with black eyes, and predisposed to ovarian neoplasms.[9] The Wv/Wv homozygous mice contain less than 1% of the

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normal number of oocytes at birth, and the remaining oocytes are depleted by ~8 weeks of age.[9] Consequently, ovulation ceases, and an increase in pituitary gonadotropins follows because of the lack of feedback inhibition that is normally mediated through progesterone released from the corpora lutea.[10] The females recapitulate and exaggerate the postmenopausal alterations in ovarian morphology and develop ovarian tubular adenomas.[9] Elevated levels of gonadotropins are believed to be the causative factor of the ovarian neoplasm.[11,12] The ovarian lesions in the Wv mice are known as complex tubular adenomas.[9] These ovarian tumors are generally benign and seldom develop malignant features. Nevertheless, the causative factors, the depletion of germ cells, and subsequent increase in gonadotropins, mimic the condition of perimenopausal women, in which gonadotropin levels are elevated and the risk for ovarian cancer is highest.[13] In addition, the ovarian lesions of the Wv/Wv mice have particular resemblance to morphological changes found in ovaries of women with an increased risk for ovarian cancer. These changes include surface papillomatosis, deep invaginations, and cystadenomas and are considered preneoplastic lesions. Thus, the Wv/Wv mice may constitute a model to investigate how ovulation and gonadotropin stimulation during postmenopause act as etiological factors in ovarian cancer development.

Cyclooxygenase (Cox)-2, encoded by the prostaglandin synthase 2 (*ptgs2*) gene, is a key downstream component of gonadotropin-stimulated signaling in ovulation, and Cox-2 knockout mice are anovulatory.[14–16] The role of Cox-2 in ovulatory rupture of the ovarian surface is similar to an inflammatory process.[17] Cox-2 is often overexpressed in human cancers,[18,19] and suppression of Cox-2 by either genetic or pharmacological approaches has been shown to reduce colon tumor development in mouse models[20–23] and in humans.[24] Inhibition of Cox enzymes by nonsteroidal anti-inflammatory drugs also seems to reduce ovarian cancer risk,[25–27] and a possible mechanism is that inhibition of Cox-2 may reduce the cancer-promoting activity of the inflammation-like ovulatory processes that are stimulated by gonadotropins.[28] In this study, we investigated the role of Cox-2 in the development of ovarian tumors in the germ cell-deficient Wv mice, which model postmenopausal ovarian biology.

Materials and Methods

Generation and Genotyping of Wv:Cox-2 Mutant Mice

An inbreeding colony was established by crossing Wv/+ and Cox-2 (+/−) mice in the C57BL/6J background (Jackson Laboratory, Bar Harbor, ME). Littermates with Wv/+:Cox-2 (+/−) genotype were further intercrossed to generate mutant and control mice for analysis. The Wv genotypes of the resulting progeny were identified by the coat color: white, gray with ventral/dorsal spots, or black represents Wv/Wv, Wv/+, or Wv (+/+), respectively. Cox-2 genotypes were verified by polymerase chain reaction (PCR) analysis using the genomic DNA isolated from mouse tails with the following primers[29]: Cox-2 forward: 5′-GCCCTGAATGAACTGCAGGACG-3′ and reverse: 5′-ACCTCTGCGATGCTCTTCC-3′; Neo forward: 5′-GCCCTGAATGAACTGCAGGACG-3′ and reverse: 5′-CACGGGTAGCCAACGCTATGTC-3′. PCR reactions were set up in a 25-μl total volume with a final concentration of 2.5 U of platinum *Taq*DNA polymerase (Invitrogen, Carlsbad, CA), 1× PCR buffer (Invitrogen), 1.5 mmol/L $MgCl_2$, 0.2 μmol/L primers, 0.2 mmol/L dNTP mixtures (Promega, Madison, WI), and 15 to 20 ng genomic DNA. Cycling conditions were followed as that described by the Jackson Laboratory. In brief, the PCR reactions were performed with 35 cycles consisting of melting at 94°C for 30 seconds, annealing at 66°C for 1 minute, and extension at 72°C for 1 minute. At the end of the PCR reactions, the mixtures were kept for an additional 2 minutes at 72°C and stored at 10°C until analysis. PCR products were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide. The presence of wild-type and neo alleles corresponds to 857-bp and 500-bp products, respectively. Approximately 30 to 40% of homozygous Cox-2 mutant mice were lost during the preweaning stage,[29] and only limited numbers (28 females were obtained and analyzed so far) of mice with the Wv/Wv:Cox-2 (−/−) genotypes were obtained.

Analysis and Quantitation of Tumor Phenotype

The ovaries of 4- to 5-month-old mice with Wv/Wv, Wv/Wv:Cox-2 (+/−), or Wv/Wv:Cox-2 (−/−) genotypes were harvested for histological analysis of ovarian tumor phenotype. The largest cross-section of an ovary was stained with cytokeratin-8 to identify epithelial components, and a digital image was recorded. The degree of tumor phenotype was defined as the percentage of the ovary penetrated by the cytokeratin-8-positive epithelial tubular structure, which was quantitatively calculated by laying a 20 × 20 grid over the ovarian image to divide the ovary into 200 to 300 squares. Grids positive and negative for internal epithelial lesions were counted independently by two noninvolved persons (student assistants) and used to calculate the percentage of ovaries infiltrated by the tubular adenomas.

Preparation of Ovarian Lysate and Western Blotting

Mouse ovaries were snap-frozen on surgical removal and maintained at −80°C until the tissues were used for protein analysis. To prepare ovarian lysates, frozen tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 mmol/L Tris, pH 7.2, 100 μmol/L sodium orthovanadate, and 50 mmol/L NaF) containing protease inhibitors (Boehringer Mannheim, Indianapolis, IN) using a Mini-

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BeadBeater (BioSpec Products, Bartlesville, OK) at 5000 rpm, 20-second interval for a total of 4 minutes. The homogenate was centrifuged at 10,000 × *g* for 10 minutes to remove the particulate material. The protein concentration in the supernatant was measured using the DC protein assay (Bio-Rad, Hercules, CA).

For Western blotting, an aliquot of the total ovarian lysate was separated by electrophoresis on a 4 to 12% gradient gel and electrotransferred onto a polyvinylidene difluoride membrane. Membranes were incubated with antibodies against either Cox-1 or Cox-2 (Cayman Chemicals, Ann Arbor, MI), or β-actin (Sigma, St. Louis, MO) followed by horseradish peroxidase-labeled secondary antibodies (Sigma). The signals were revealed using a chemiluminescence detection system (Pierce, Rockford, IL). Ovarian lysate from Cox-1 (−/−) mice (obtained from Dr. Robert Langenbach) was used to confirm the specificity of Cox-1 antibodies.

AQ: E

Immunohistochemistry

Ovaries were fixed in buffered formalin and embedded in paraffin. The paraffin blocks were cut into 5-μm-thick sections that were placed on positively charged slides. The sections were dewaxed in xylene and hydrated through graded ethanol. Heat-induced antigen retrieval was then performed in 10 mmol/L sodium citrate (pH 6.0) in a microwave initially at high-power setting (no. 10) for 2 minutes followed by low-power setting (no. 2) for 10 minutes. The endogenous peroxidase activity was blocked by immersing the slides in 3% H_2O_2 in methanol for 15 minutes. After 30 minutes of incubation with blocking serum, slides were incubated with rat monoclonal anti-Troma-1/cytokeratin-8 antibodies (Developmental Studies Hybridoma Bank from The University of Iowa, Ames, IA) at 1:600 dilution, Cox-2 rabbit polyclonal antibodies (Cayman Chemical) at 1:600 dilution, Cox-1 rabbit polyclonal antibodies (Cayman Chemical) at 1:500 dilution, or F4/80 rat polyclonal antibodies (Serotec Ltd., Kidlington, Oxford, UK) at 1:200 dilution at 4°C overnight followed by incubating with goat anti-rat horseradish peroxidase-labeled secondary antibodies (BD Pharmingen, Franklin Lakes, NJ) at 1:100 dilution for cytokeratin-8, anti-rabbit labeled polymer horseradish peroxidase (DAKO, Carpinteria, CA) at 1:100 dilution, or biotinylated anti-rat at 1:200 dilution for F4/80 for 35 minutes at room temperature. Diaminobenzidine was used as the chromogen for the immunoperoxidase reaction. The slides were counterstained with hematoxylin and mounted in 50:50 xylene/Permount.

Prostaglandin E_2 Assay

The prostaglandin E2 (PGE_2) level in the ovarian lysates was measured using an enzyme-immunoassay kit (Cayman Chemical). This assay is based on the competition between PGE_2 and a PGE_2 acetylcholinesterase conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibodies. This antibody-PGE_2 complex binds to goat polyclonal anti-mouse IgG that has been attached to the plate. In brief, ovarian lysates together with enzyme-immunoassay buffer, PGE_2 tracer, and antibody were added into the plate. The mixtures were incubated at 4°C overnight. The plates were washed to remove the unbound reagents and developed with the Ellman's reagent (substrate), and the absorption was measured at 412 nm. The amount of PGE_2 in the sample was determined from a standard curve.

FSH Assay

At the time of sacrifice, 100 to 200 μl of sera were collected from each animal and stored at −80°C until testing. The FSH level in the serum was measured by radioimmunoassay through custom service from the National Hormone and Peptide Program, Harbor-UCLA Medical Center (Torrance, CA).

Drug Administration

Four-week-old female Wv/Wv mice, weighing ~14 g, were randomly allocated into three groups: nontreated control (n = 5), indomethacin-treated (n = 7), and celecoxib (Celebrex)-treated groups (n = 8). Celecoxib was administrated through feeding with AIN 76A diet (Dyets Inc., Bethlehem, PA) containing 1500 ppm celecoxib (Fox Chase Cancer Center Pharmacy). Indomethacin was introduced through drinking water (6 mg/ml). During the study, mice were permitted free access to the diet and drinking water. All of the mice were inspected once daily to monitor their general health status. Body weight was measured once per week throughout the experiments. Optimal dosage was estimated to be 100 μg/day for celecoxib and 30 μg/day for indomethacin. Dosages double these amounts resulted in some toxicity such as reduced weight or activity/alertness in the female Wv/Wv mice. Mice were sacrificed at ~4 months of age. Ovarian tissues were collected and subjected to histopathological examination.

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Statistical Analysis

Basic and standard analytical procedures were applied to examine the statistical significance of the data. Differences in proportions were evaluated by the χ^2 or the Fisher exact test, as appropriate. Student's t-test was used to compare the differences in means between two groups. Statistical significance was considered as $P < 0.05$. All P values are two-sided.

Results

Ovarian Surface Epithelia Undergo Morphological Transformation and Tumorigenesis in Wv Mice

We have maintained a colony of Wv mice by inbreeding for the last 3 years. Of the more than 200 mature (3 months or older) Wv/Wv females examined, all exhib-

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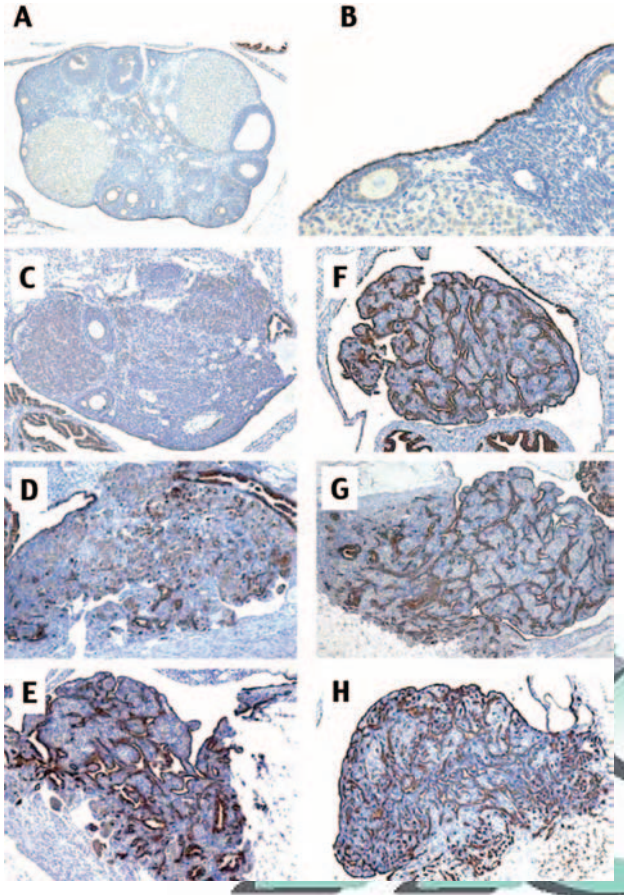

Figure 1. Development of tubular adenomas in Wv/Wv mice. Ovarian morphology was compared between wild-type littermates (**A** and **B**) and Wv/Wv (**C–H**) mice. Cytokeratin-8 (Troma-1) staining was used to highlight epithelial cells. **A:** An ovary from wild-type control at 4 months of age and **B:** at higher magnification. Representative examples of ovaries from Wv/Wv mice at the age of 1 month (**C**), 2 months (**D**), 3 months (**E**), 4 months (**F**), 5 months (**G**), and 9 months (**H**) are shown. At least five mice at each time point were examined. Original magnifications: ×40 (**A**, **C–H**); ×100 (**B**).

ited ovarian tubular adenomas, whereas none were observed in the wild-type littermates. The progressive changes in ovarian morphology were documented, and representative examples are shown in Figure 1. As a comparison, the wild-type ovary contains developing follicles of all stages and multiple corpora lutea (Figure 1A). The surface epithelia lining the perimeter of the ovary are the only cells positive for cytokeratin-8, a marker of epithelial cells. Higher magnification reveals the well-organized surface epithelial cells and interstitial stroma cells in the ovarian cortex (Figure 1B). In Wv/Wv mice at 1 month of age, few follicles can be observed in the ovary although the cortex is still enveloped by a smooth layer of cytokeratin-8-positive surface epithelial cells (Figure 1C). We began to observe the presence of tubule-like structures in the ovarian cortex of the Wv/Wv mice by 2 months of age (Figure 1D). By the end of 3 months, the entire ovary was replaced by tumorous lesions. Positive staining for cytokeratin-8 indicates the epithelial origin of the tubular structures (Figure 1E). At 4 and 5 months, the phenotype of tubular adenomas became more complex as shown by the increased penetration and branching of the epithelial tubules (Figure 1, F and G), together with the appearance of scalloping and crowding of epithelial cells into multiple cell layers. We have analyzed the Wv/Wv ovaries for up to 1 year of age when the entire ovaries were completely permeated with the tumor cells. The lesions are somewhat more complex and intense in older mice, but the tumor cells have not further expanded into large masses or acquired malignant features (Figure 1H). Although the ovarian lesions in the Wv mice distribute throughout the ovarian stroma, and are known as stromal tubular adenomas,[9] the contiguous connection to ovarian surface epithelium is evident. This is especially pronounced in cases of early ovarian lesions in younger (7 to 10 weeks) mice when only a few lesions have developed. Obvious surface origination of the epithelial lesions can be observed (Figure 2A, arrow): the tubular structure is contiguous with the monolayer of the surface epithelium (Figure 2B). We conclude that most if not all of the tubular adenomas in Wv/Wv ovaries are derived from ovarian surface epithelial cells. The majority of the lesions either exhibit inclusion cyst-like structures (Figure 2C) or resemble surface deep invaginations/papillomatosis (Figure 2D). Although dysplastic morphology is evident in some epithelial compartments of the tubular adenomas (Figure 2, E and F), the ovarian epithelial tubular structures in the Wv mice are considered benign tumors and the lesions are confined to ovarian tissues and do not become metastatic. Histopathological evaluation also indicated their benign cytological morphologies without evident mitotic figures. These observations are consistent with the notion that the benign epithelial tubular adenomas are caused by the stimulation of the reproductive hormones, gonadotropins, rather than oncogenic alterations. Indeed, we confirmed that serum FSH, a key gonadotropin, is greatly increased (~10-fold) in female Wv/Wv mice (Figure 2G). The magnitude of FSH increase is very similar to the elevation found in menopausal women.[1] Thus, it seems that the Wv/Wv mouse model closely mimics the changes in ovarian physiology (follicle depletion), endocrine factor (hormonal increase), and ovarian pathology (morphological changes) in menopausal women. In the investigation of potential ovarian mediators of gonadotropins, we found that ovarian Cox-2 protein levels are dramatically increased in Wv/Wv mice compared with those of control littermates (Figure 2H). Accordingly, the ovarian PGE_2 level is threefold to fivefold higher in Wv/Wv mice (Figure 2I).

Wv Mouse Tumor Phenotype Is Suppressed by a Reduction of Cox-2 Gene Dosage

We investigated whether Cox-2 plays a role in the formation of tubular adenomas in Wv mice in which the elevated gonadotropins likely stimulate Cox-2 expression. Cox-2 deficiency was introduced into the Wv mouse colony by crossing Wv/+ with Cox-2 (+/−) mice, and a new inbred colony was established by

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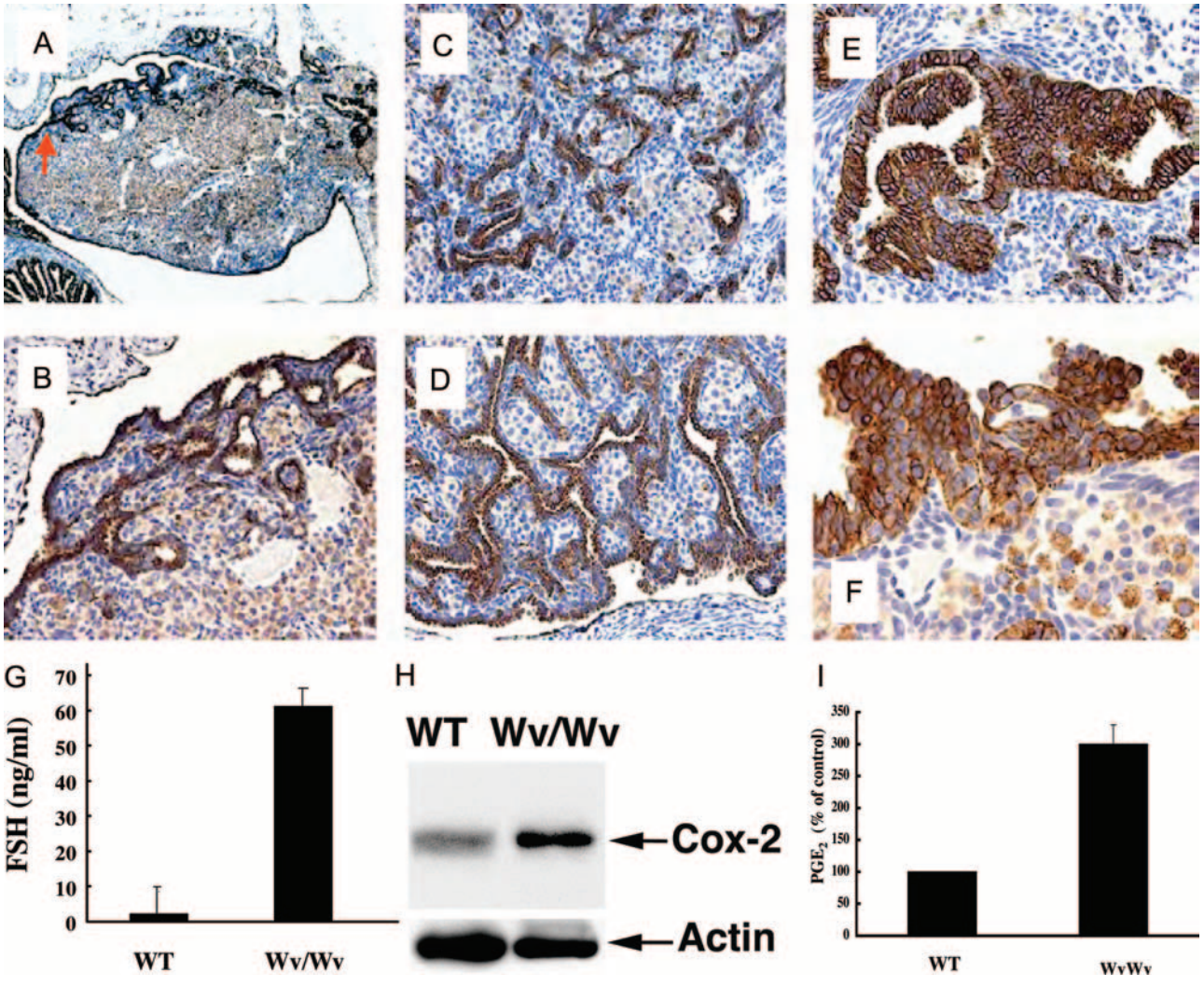

Figure 2. Ovarian morphological and physiological changes in Wv/Wv mice. Ovarian tissues were harvested and subjected to histological analysis. Cytokeratin-8 staining was used as a marker for epithelial cells. **A:** An example of an ovary from a 7-week-old Wv/Wv mouse is shown for the surface epithelial origin of the tubular adenoma structure. The **arrow** indicates the contiguous links between surface epithelium and the tubular epithelial structure, which are stained for cytokeratin-8, and the area is shown at a higher magnification in **B**. **C** and **D:** Two representative examples of tubular adenomas; and **E** and **F:** two examples of dysplastic epithelial cells from ovarian tubular adenomas of 4-month-old Wv/Wv mice. **G:** Serum was collected from four each of wild-type and Wv/Wv 4-month-old female mice to determine FSH levels in triplicate. Averages of triplicate with SDs are shown. Two-sided Student's *t*-test indicates the difference is statistically significant ($P < 0.001$). **H** and **I:** Ovaries ($n = 10$) from five female mice each, of either Wv/Wv or wild-type littermates at 4 months of age, were collected, dissected to remove the surrounding fat tissues, and pooled. The tissues were homogenized in RIPA buffer and the lysate was used for measurement of Cox-2 protein by Western blot (**H**) and PGE_2 level by EIA (**I**). Actin level was used as a protein loading control in Western blotting. Averages of triplicate with SDs are shown for PGE_2 levels. Two-sided Student's *t*-test indicates the difference is statistically significant ($P < 0.001$). Examples of ovarian morphology are shown by H&E staining in Supplemental Figure 2 (see *http://ajp.amjpathol.org*). Original magnifications: ×40 (**A**); ×200 (**B–F**).

crossing Wv/+:Cox-2 (+/−) siblings. Progenies homozygous for Wv and all genotypes of Cox-2, (+/+), (+/−), or (−/−), were examined for ovarian morphology at ~4 months of age (Figure 3), when the tubular adenoma phenotype seems to be fully presented and fairly uniform in the Wv/Wv mice. The ovarian tubular adenoma phenotype in the 70 Wv/Wv:Cox-2 (+/+) mice produced in this colony was essentially identical to that found in more than 200 female mice of the original colony before introduction of the Cox-2 mutation: all of the ovaries exhibited severe complex tubular adenomas that permeated the entire organ. We observed a significant alleviation of ovarian lesions in the Wv/Wv:Cox-2 (+/−) ovaries analyzed (Figure 3, D–F), although the degree to which the tumor phenotype was suppressed varied greatly (Figure 3G). Some ovaries completely lacked epithelial tubular structures inside the cortex (Figure 3, D and F), and some contained only a small number of epithelial tubular structures (Figure 3E). Unlike wild-type ovaries, these Wv/Wv:Cox-2 (+/−) ovaries lacked apparent follicles or corpora lutea. Thus, hemizygous reduction of the Cox-2 gene resulted in a complete (Figure 3, D and F) or partial (Figure 3E) rescue from the epithelial adenoma phenotype. Unexpectedly, of the 10 ovaries from mice of Wv/Wv:Cox-2 (−/−) genotype, only 3 ovaries exhibited a significant (50% area) reduction in the tubular adenoma phenotype (Figure 3, A and G). All other

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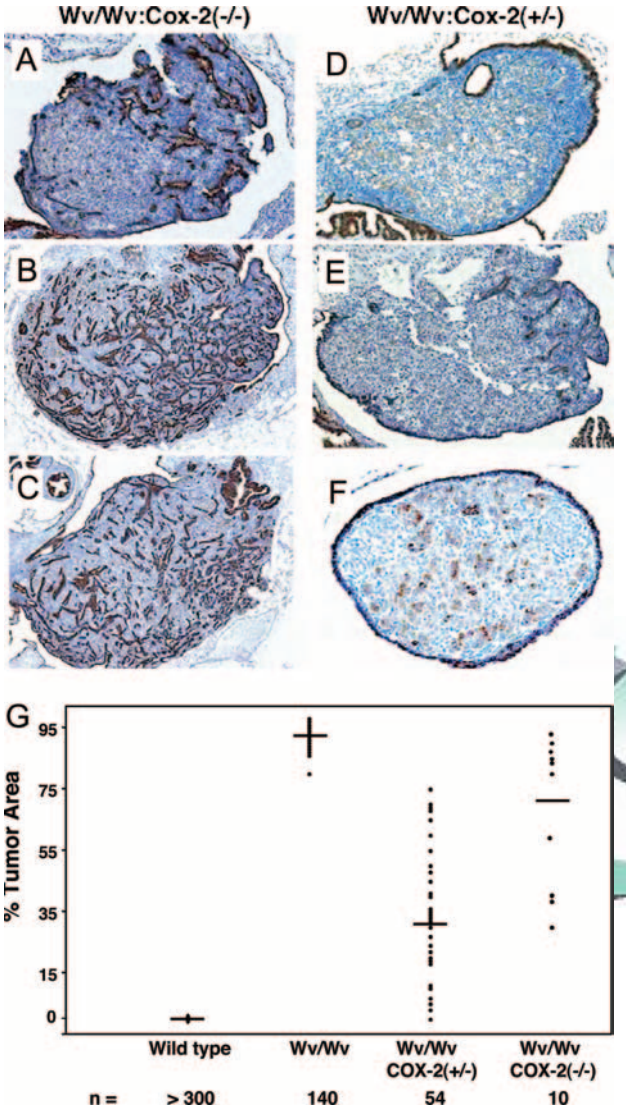

Figure 3. Rescue of Wv/Wv mouse ovarian tubular adenoma phenotype by the Cox-2 knockout. The ovarian morphology from littermates of the Wv and Cox-2 knockout inbred colony was compared. Cytokeratin-8 (Troma-1) staining was used to identify epithelial cells. Representative examples of ovaries from 4.5-month-old mice of Wv/Wv:Cox-2 (−/−) (**A–C**) and Wv/Wv:Cox-2 (+/−) (**D–F**) genotypes are shown. The image shown in **A** is the example of ovary (n = 10) from Wv/Wv:Cox-2 (−/−) mice with significant reduction in ovarian lesions. **G:** The degree of tumor involvement of each ovary was estimated and the distribution is plotted. n indicates the number of ovaries analyzed. The mean value for ovarian tumor involvement is 0% for wild-type, 93% for Wv/Wv, 32% for Wv/Wv:Cox-2 (+/−), and 70% for Wv/Wv:Cox-2 (−/−) genotypes. Student's t-tests showed $P < 0.005$ for Wv/Wv versus Wv/Wv:Cox-2 (+/−), and $P < 0.05$ for Wv/Wv versus Wv/Wv:Cox-2 (−/−), indicating a statistically significant difference. The difference between Wv/Wv:Cox-2 (+/−) and Wv/Wv:Cox-2 (−/−) also is statistically significant ($P < 0.001$). Examples of ovarian morphology are shown by H&E staining in Supplemental Figure 3 (see *http://ajp.amjpathol.org*). Original magnifications, ×40.

ovaries showed ovarian morphology indistinguishable from that of Wv/Wv:Cox-2 (+/+) genotype (Figure 3, B and C). The mean value for the area of the ovary covered by lesions is 93% for Wv/Wv, 32% for Wv/Wv:Cox-2 (+/−), and 70% for Wv/Wv:Cox-2 (−/−) genotypes (Figure 3G). Thus, a reduction in Cox-2 gene dosage rescued the ovarian epithelial morphological alteration, but deletion of both copies was less sufficient in reversing the adenoma phenotype in Wv/Wv mice.

Cox-2-Null Deletion Causes a Compensatory Increase in Cox-1 Expression in Ovaries

Measurement of prostaglandin levels in ovaries of these mice showed that Wv/Wv mice exhibited an increase of ovarian PGE_2 (Figure 4A) and Cox-2 (Figure 4B) levels F4 over the wild-type littermates, to 4.2- and 2.1-fold, respectively. The expression of the Cox-1 protein was also elevated over that of wild type (3.5-fold). This increase in the Cox-1 level is unexpected because it is commonly accepted that Cox-1 is a housekeeping gene and generally not regulated.[30] However, Cox-1 expression was previously found increased in human ovarian cancer[31,32] and in ovarian tumors found in a variety of mouse ovarian cancer models.[33]

Ablation of one allele of Cox-2 lowered the amount of PGE_2 produced in the ovaries by 45% (compare Wv/Wv:Cox-2 (+/−) to Wv/Wv) (Figure 4A). The prostaglandin level in Wv/Wv:Cox-2 (−/−) mice was higher than that of Wv/Wv:Cox-2 (+/−) and similar to that of Wv/Wv mice (Figure 4A). The ovarian Cox-1 protein amount in Wv/Wv:Cox-2 (−/−) was determined to be 2.8-fold of those in Wv/Wv or Wv/Wv:Cox-2 (+/−) mice (Figure 4C). Presumably, the Cox-2 homozygous deletion causes a compensatory increase in Cox-1 and total prostaglandin level. The Cox-1 compensation was also observed in non-Wv Cox-2-null mice (Figure 4, D and E). In the ovarian extract, the PGE_2 level was increased 25% in Cox-2 homozygous knockout mice, although ovarian PGE_2 was 20% less in Cox-2 hemizygous mice (Figure 4D). Accordingly, ovarian Cox-1 protein was increased 2.1-fold in Cox-2 (−/−) mice but remained unchanged in Cox-2 (+/−) mice (Figure 4E). The expression of Cox-1 and Cox-2 was analyzed by immunostaining in wild-type and Wv/Wv ovaries (Figure 4F). Weak Cox-1 staining distributed evenly throughout the wild-type ovary, and Cox-2 staining was observed in stromal and follicular cells but not in surface epithelial cells. In Wv/Wv ovaries, both epithelial and stromal cells stained strongly for Cox-1 and Cox-2 (Figure 4F).

We studied the epithelial and stromal compartments to determine whether tumor phenotype and Cox-2 expression correlated with inflammation in the ovaries. Infiltration of neutrophils and eosinophilic cells was rarely observed in Wv mouse ovaries and tumors, and no evidence of acute inflammation was found. We also examined macrophage density that associates with chronic inflammation by staining for the F4/80 marker (Figure 5, A F5 and B). F4/80 staining indicated a robust increase in the appearance of macrophages in the tumorous Wv/Wv ovaries over those of the wild-type littermates (Figure 5A). Consistently, the macrophage number was reduced in the recovered ovaries of Wv/Wv:Cox-2 (+/−) genotype but not of the Wv/Wv:Cox-2 (−/−) genotype (Figure 5, A and C). Moreover, in nontumorous wild-type and Cox-2

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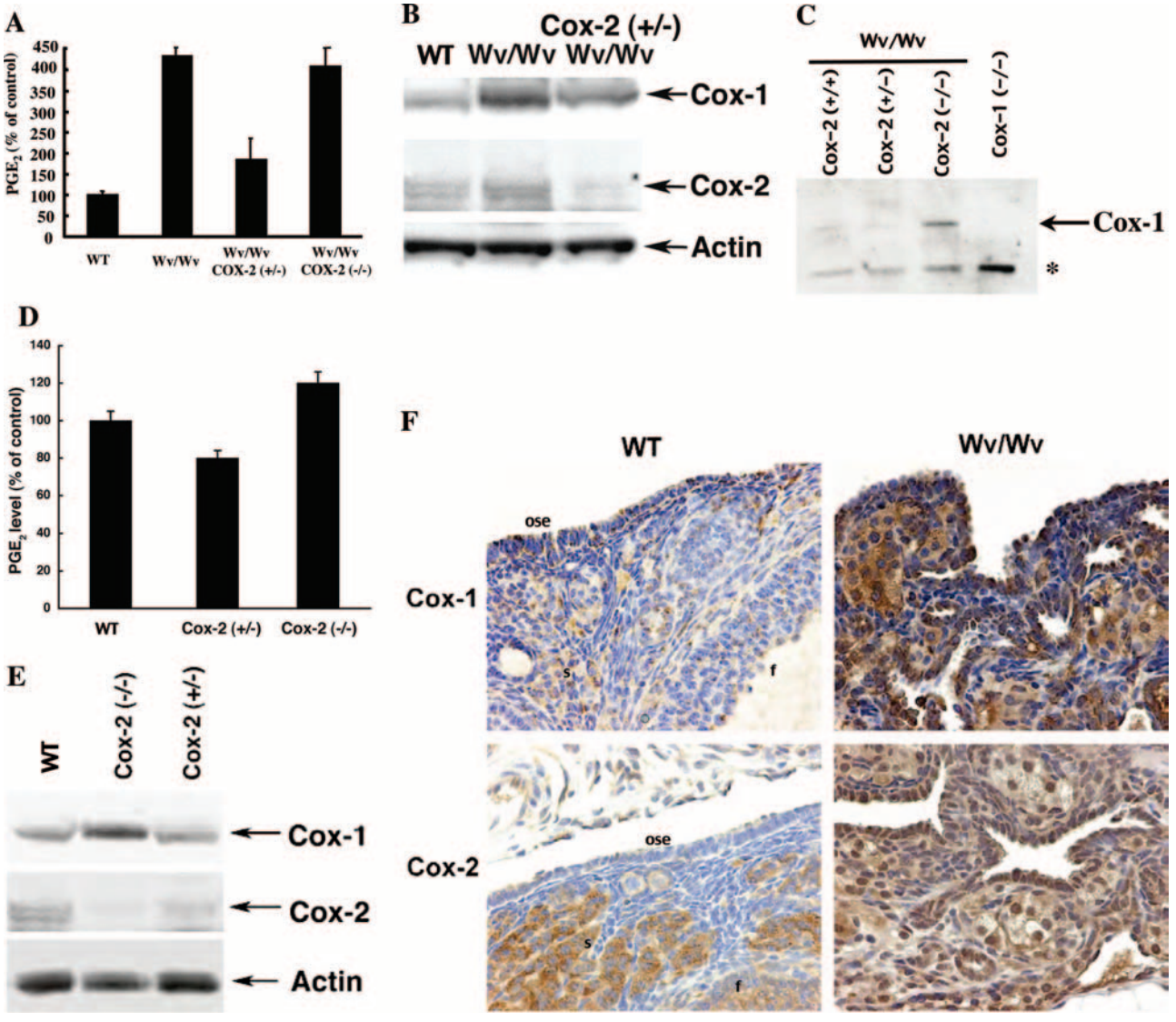

Figure 4. Ovarian PGE_2, Cox-1, and Cox-2 levels in Wv and Cox-2 mutant mice. Ovaries ($n = 4$ to 10) from female mice, of either Cox-2 (−/−), Cox-2 (+/−), Wv/Wv, Wv/Wv:Cox-2 (+/−), or wild-type littermates at 4 months of age were collected and pooled. The tissues were homogenized in RIPA buffer and the lysate was used for measurement of PGE_2 level by EIA. Relative values compared with wild type (WT) are reported as the percentage of the wild-type control, which is 3.4 pg/μg protein. **A:** Three experiments were performed to obtain the representative result and the averages of triplicate with SDs are shown. Two-sided Student's *t*-test indicates the difference is statistically significant for the comparisons between WT and Wv/Wv ($P < 0.0001$), WT and Wv/Wv:Cox-2 (+/−) ($P < 0.001$), WT and Wv/Wv:Cox-2 (−/−) ($P < 0.0028$), Wv/Wv and Wv/Wv:Cox-2 (+/−) ($P < 0.0001$), and Wv/Wv:Cox-2 (+/−) and Wv/Wv:Cox-2 (−/−) ($P < 0.0002$), but the difference does not reach statistical significance comparing Wv/Wv and Wv/Wv:Cox-2 (−/−) ($P > 0.12$). **B:** Cox-1 and Cox-2 proteins in the ovarian lysate from a pool of 10 ovaries of each genotype were determined by Western blot with actin as loading control. **C:** One of two representative experiments was shown for the determination Cox-1 protein from two ovaries (one mouse) by Western blot. Ovarian lysate from a Cox-1 (−/−) mouse was used for control for the specificity of the anti-Cox-1 antibodies. *Nonspecific protein band that served as a protein loading control. **D** and **E:** Ovaries ($n = 10$) from five mice each of either Cox-2 (−/−), Cox-2 (+/−), or wild-type (WT) littermates at 4 months of age were collected for PGE_2 assays (**D**) and Western blot analysis for Cox-1 and Cox-2 proteins with actin as a loading control (**E**). Two-sided Student's *t*-test indicates the differences between the PGE2 levels are statistically significant comparing WT and Cox-2 (+/−) ($P < 0.02$) or WT and Cox-2 (−/−) ($P < 0.04$). **F:** Ovaries from wild-type and Wv/Wv genotypes were stained for Cox-1 or Cox-2. Original magnifications, ×200.

(−/−) ovaries, macrophages were distributed in the cortex and stromal compartments but were absent in or near epithelial cells. In the ovarian tumors from Wv/Wv mice, macrophages often infiltrated into the epithelia (Figure 5B, red arrow). The relative macrophage density was quantitated as shown in Figure 5C. The correlation of macrophage density with tumor phenotypes suggests that inflammatory reactions promote the tubular adenoma development in Wv ovaries.

Pharmacological Inhibitors of Coxs Effectively Prevent Ovarian Epithelial Transformation and Tumorigenesis

We then tested if the effect of reducing the Cox-2 gene dosage on ovarian tumor phenotype can be achieved by using pharmacological agents. Based on previous studies and our own toxicity study specifically in Wv/Wv fe-

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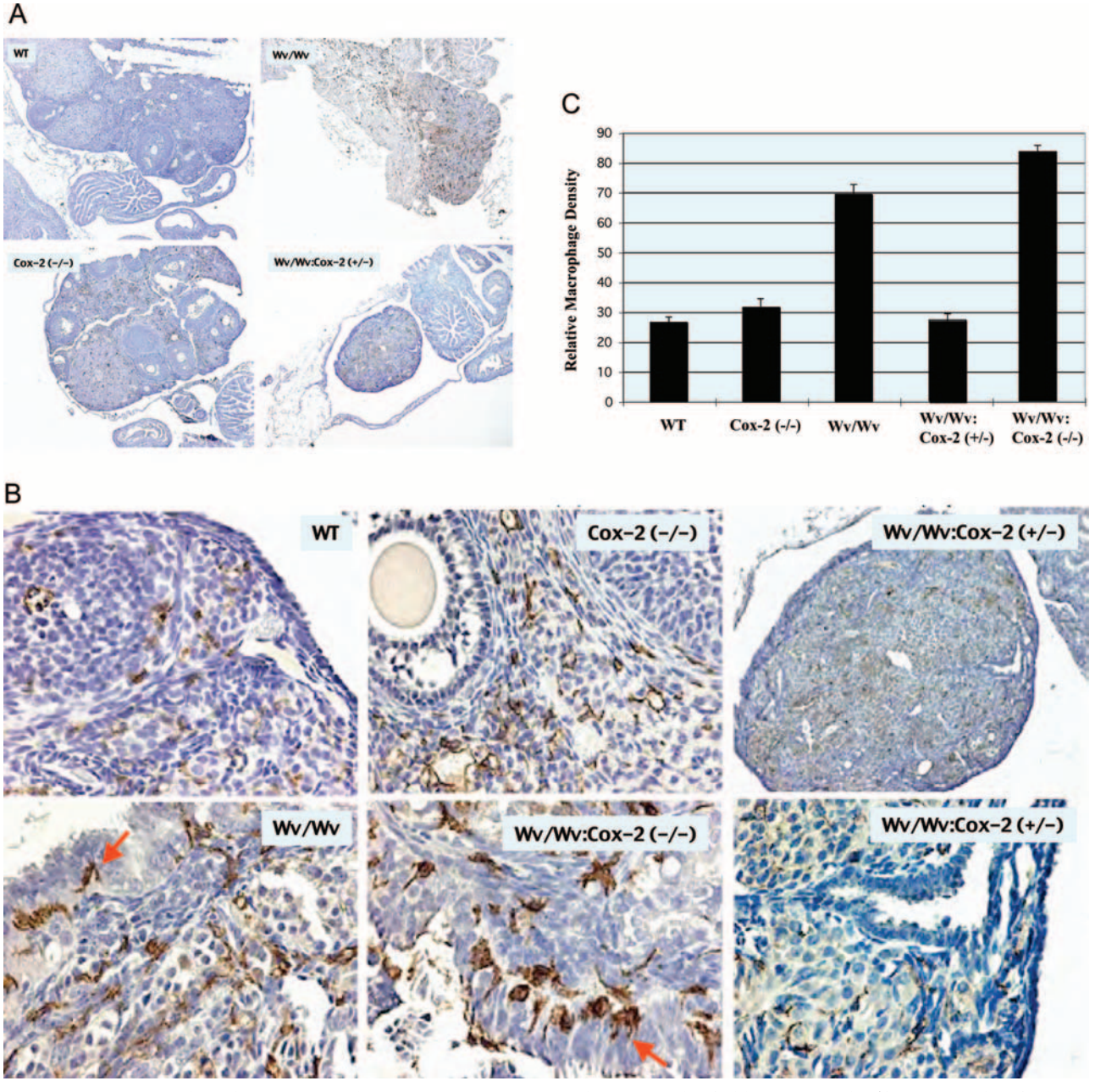

Figure 5. Ovarian macrophage density in Wv and Cox-2 mutant mice. **A:** Macrophages were identified by staining with F4/80. **B:** Examples of a higher magnification of ovaries stained with the F4/80 macrophage marker are shown. The **arrow** indicates macrophages infiltrating epithelia. **C:** Macrophage density was quantitated by counting five fields each of three ovaries from each genotype. ose, ovarian surface epithelial; s, stroma; f, follicle. Averages of five countings with SDs are shown. Student's *t*-test indicates that macrophage numbers are not statistically significant between WT, Cox-2 (−/−), and Wv/Wv:Cox-2 (+/−) ($P > 0.05$), but when the values of these three genotypes compared with those of Wv/Wv or Wv/Wv:Cox-2 (−/−), the difference is statistically significant ($P < 0.001$). Original magnifications: ×40 (**A**); ×200 (**B**).

male mice, nontoxic dosages of celecoxib, a Cox-2-specific inhibitor, and indomethacin, a nonselective COX inhibitor, were determined to be 200 and 30 μg/day, respectively. When the Wv/Wv female mice received these inhibitors starting at 4 weeks of age, a significant reduction in tumor phenotype was observed at the age of 3 months in all ovaries, and some ovaries were devoid of tubular adenomas (Figure 6). In three of eight Wv/Wv mice, celecoxib feeding prevented the ovarian tumor phenotype; and in the indomethacin-treated group, five of seven mice were rescued. All ovaries of the five control Wv/Wv littermates that were maintained identically but did not receive inhibitors showed ovarian tubular adenomas. Only the comparison between controls and indomethacin-treated mice in this experiment reaches statistical significance. However, considering that ovarian tumors were found in all of the more than 300 Wv female mice analyzed previously, the observed reduction in tumor development by both celecoxib and indomethacin is likely significant. Thus in this preliminary study including only a small number of animals, we found that Cox inhibitors are able to prevent ovarian epithelial morphological

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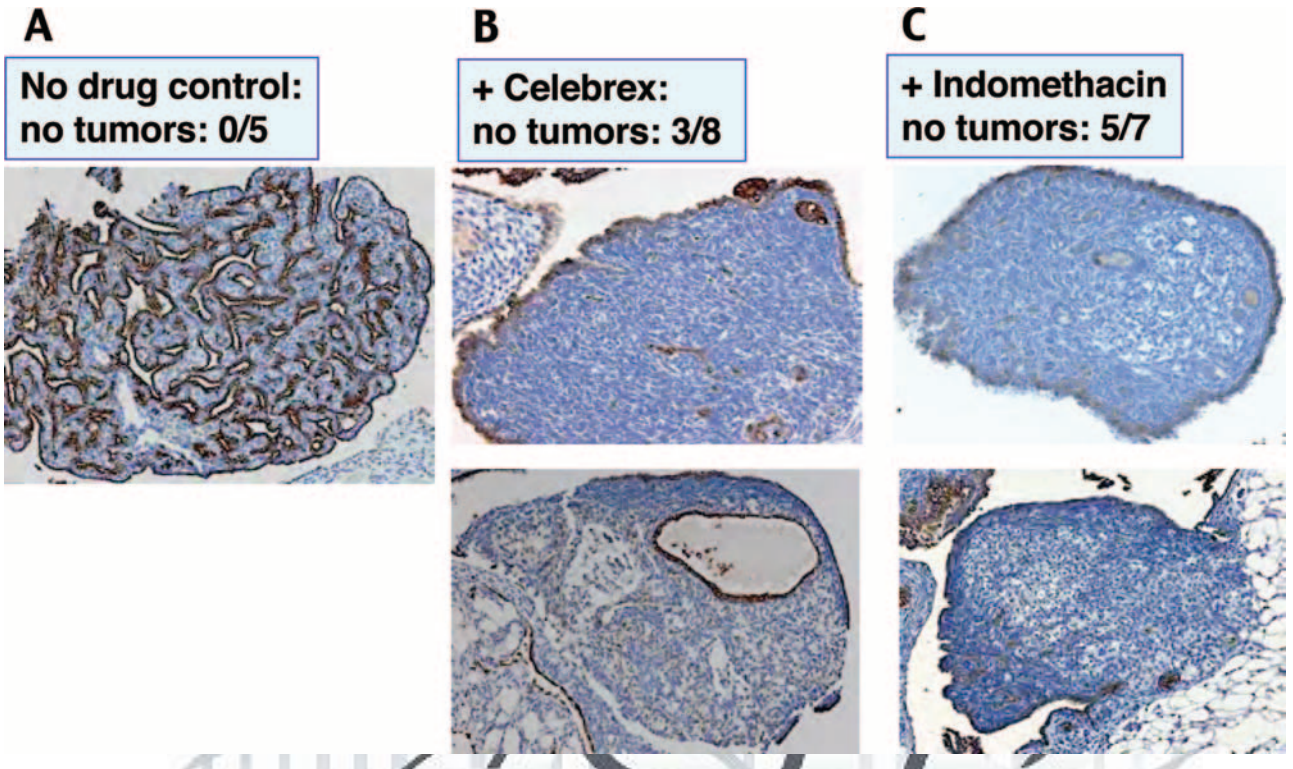

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Figure 6. Rescue of Wv/Wv mouse ovarian tubular adenoma phenotype by celecoxib (Celebrex) and indomethacin administration. The representative ovarian morphology from Wv/Wv mice controls and those fed with celecoxib or indomethacin was compared. Cytokeratin-8 (Troma-1) staining was used to identify epithelial cells. The *P* values generated from the χ^2 test (or Fisher's exact test) are 0.2308, 0.0278, and 0.3147, respectively, for the comparisons between celecoxib treatment versus control (**A**), indomethacin treatment versus control (**B**), and celecoxib versus indomethacin treatment (**C**). Thus in this experiment only the difference between indomethacin treatment versus control reaches statistical significance. One example of wild type and two examples of treatment with celecoxib and indomethacin are shown. Original magnifications, ×40.

transformation and tumor phenotypes. Inhibition of both Cox-1 and Cox-2 with indomethacin seems more effective than inhibition of Cox-2 alone with celecoxib, but the difference did not reach statistical significance. The number of animals used in these experiments is relatively small, and additional confirmation is needed. When indomethacin was given for a period of 1 month to Wv/Wv mice at 3 months of age when ovarian tumors were already established, the tumors were not reduced compared with controls (not shown), suggesting inhibition of Coxs prevents the development of ovarian tumors but has no suppressive effect on established tumors.

Discussion

In the present study, we demonstrate that a reduction of Cox-2 gene dosage rescued the ovarian aging phenotype of the Wv/Wv mice, whereas homozygous deletion of Cox-2 gene was less effective in reducing the formation of these epithelial lesions in the ovaries because Cox-2 elimination was accompanied by an increase in ovarian Cox-1 expression and PGE_2 synthesis. The tumor phenotype was also reduced by the use of pharmacological agents to inhibit Cox-1 and/or Cox-2. These findings suggest that increased Cox-1/Cox-2 activity contributes to the preneoplastic morphological changes of the ovarian surface epithelium, and the tumor phenotype can be reversed by a reduction of Cox gene dosage either by genetic or pharmacological approaches. The results suggest the inflammatory environment of the germ cell-depleted ovaries stimulates epithelial morphological changes and tumor development, and this circumstance provides an excellent example of how inflammation promotes cancer. This study provides a model to study the links between reproductive factors, inflammation, and ovarian tumor development.

Inflammatory Environment in Postmenopausal Ovaries

The link between inflammation and cancer is well recognized: chronic inflammation induced by infections or other chemical and pathological factors creates an inflammatory microenvironment in the tissue, which is composed of epithelial cells, stromal cells, leukocytes, and macrophages. These cells are networked by autocrine and paracrine interactions mediated by proinflammatory cytokines such as tumor necrosis factor-α and interleukin-β.[34,35] Signal pathways involving nuclear factor-κB, tumor necrosis factor-α, and Coxs are known to be involved in both inflammation and cancer. The inflammatory mediators such as proteolytic enzymes, prostaglandins, and diverse reactive oxygen and nitrogen species create a tumor-promoting environment in which transformed (altered by either genetic or epigenetic means) epithelial cells thrive and are selected to expand.[34,35]

Coxs, Cox-1 and/or Cox-2, play crucial roles in regulation of inflammation and are thought to be targets of the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs for cancer prevention.[18,19,23]

Gene knockout mice suggest that both Cox-1 and Cox-2 are dispensable for development and have verified the role of Cox-1 and Cox-2 in inflammation that counters host infection by microorganisms.[29,30] Another established physiological function for Cox activity is in reproduction, especially ovulation,[14–16] which is considered an inflammatory-like process.[17] Ovarian Cox-2 is induced by the periodical surge of gonadotropins and plays an important and necessary role in the proteolysis and tissue remodeling that precedes the ovulatory rupture of the follicle and ovarian surface to release the ova.[36] The elevated gonadotropin level in postmenopause, although it does not cause ovarian surface rupture as in ovulation, still stimulates the ovulation-like inflammatory process and Cox expression.[28] The Wv mouse model simulates the postmenopausal ovarian inflammatory conditions as indicated by the increased expression of ovarian Cox-1 and Cox-2, and macrophage infiltration.

We propose that this ovarian inflammatory environment promotes tissue remodeling and perturbation or disruption of epithelial structure, leading to epithelial morphological transformation and the development of the ovarian tubular adenomas in the Wv mice. This mechanism may explain gonadotropin stimulation as an etiological factor for an increased ovarian cancer risk. The ovarian tumor in Wv mouse also represents a unique model in which a physiological inflammatory environment promotes tumor development.

Germ Cell-Deficient Wv Mice as Models for Reproductive Factors in Ovarian Cancer Etiology

Although the ovarian tumors may invade adjacent tissues and fat pads at later stages,[9] we have not observed any shedding of tumor cells and the formation of ascites in Wv mice. Although the ovarian tubular adenomas are derived from ovarian surface epithelial cells, the histology of the tumors differs from human epithelial ovarian cancer. Thus, the Wv mouse is not a model for human malignant epithelial ovarian cancer.

Nevertheless, the ovarian epithelial morphological transformation in Wv mice resembles ovarian morphological changes associated with reproductive aging and perimenopausal gonadotropin stimulation. Most likely, the Wv mouse model mimics certain biological aspects of ovarian cancer risk associated with menopause and gonadotropin stimulation. The ovarian tumors in Wv mice are associated with excessive hormonal stimulation, but lack genetic mutations that are commonly found in human ovarian cancer, such as Ras and B-Raf mutations in borderline tumors, p53 mutations in malignant ovarian cancer, and pten mutations in the endometrial subtype of ovarian carcinomas.[37] In the last few years, a number of technical breakthroughs have led to the establishment of several mouse models for ovarian cancer. First, a genetically defined model of ovarian cancer was established by Orsulic and colleagues,[38] in which mouse ovarian surface epithelial cells were transfected with defined genetic changes such as k-Ras, v-Akt, v-myc, and so forth. The cells were then reimplanted into the ovarian bursa and malignant ovarian tumors developed. Using the MIS II R promoter, a mainly ovarian-restricted transcript, Connolly and colleagues[39] developed the T-antigen transgenic line that develops malignant bilateral ovarian tumors. Presumably, T-antigen expression results in the inactivation of both p53 and Rb. Indeed, using adenoviral delivery of cre to ovaries of mice with floxed p53 and Rb, Flesken-Nikitin and colleagues[40] demonstrated the development of malignant ovarian tumors when both p53 and Rb are deleted. Most recently, mice with conditional expression of K-ras and deletion of pten in ovarian surface epithelial cells were found to develop endometriosis and endometrioid carcinomas.[41] Because both mutations are present in endometriosis and endometrioid ovarian cancer in humans, this model seems to recapitulate the genotype and histomorphology associated with the human disease. There are likely additional ovarian cancer animal models that are not mentioned here. These genetic models have provided persuasive evidence for the relevance of these mutations in ovarian carcinogenesis, but nevertheless have not yet incorporated components related to the etiology of ovarian cancer.

To reconcile the ovarian etiology related to gonadotropin stimulation and postmenopausal biology with genetic mutations in the development of ovarian cancer, it may be proposed that the postmenopausal gonadotropin stimulated epithelial proliferation and morphological transformation may select and promote the expansion of cells with genetic mutations. Mice with a p53 mutation alone do not develop ovarian epithelial tumors, even when ovaries with mutant p53 are transplanted into wild-type hosts to bypass the development of sarcomas and lymphomas in the p53 mutant background.[42] As a prediction, if additional genetic mutations (such as a p53 mutation) are added to the Wv/Wv females, malignant ovarian carcinomas may develop. Preliminary results of these experiments in our laboratory are very suggestive, but a thorough analysis of these mice with compound genetic mutations will take its course.

Implication of the Cox-2 Gene Dosage Effect

Unexpectedly, a reduction of Cox-2 gene dosage by heterozygous deletion is more effective than homozygous deletion in preventing the ovarian epithelial morphological change and the formation of tubular adenomas in Wv/Wv mice. This seems attributable to a compensatory increase in ovarian Cox-1 when Cox-2 is completely eliminated. Although a compensation between the expression of Cox-1 and Cox-2 was not observed in Cox-2 gene knockout mice initially,[14,29] it has since been noted,[43] and Cox-1 can substitute for Cox-2 in ovulation in certain genetic backgrounds.[44] Thus, the roles of both Cox-1 and Cox-2 in promoting ovarian tumor development sug-

gest drugs that inhibit both Coxs may be more effective than specific inhibitors of Cox-2 in reducing the development of ovarian tumors.

Moreover, a reduction of the Cox-2 gene copy number is sufficient to reduce the ovarian tumor phenotype in the Wv mice is unique, because reduction of one gene copy number seldom shows a phenotype in gene knockout mice. This dosage-dependent effect of Cox-2 in promoting ovarian tumor development may have crucial implications in strategies using inhibitors as preventive agents for ovarian cancer. It suggests that the use of a low dosage of the drugs to reduce Cox-2 (and Cox-1) activity, rather than a complete suppression of the activity, may be sufficient to reduce tumor incidence.

Interestingly, Cox-1, instead of Cox-2, was found to be overexpressed in human ovarian cancer cancer.[31,32] The increased Cox-1 expression was observed in several mouse ovarian tumor models models,[33] and we also found that Cox-1 is increased in Wv mouse ovarian tumors. Thus, Cox-2 may be important in tumor initiation, and Cox-1 increase may be important for progression and malignancy.

In summary, this study indicates that inhibition of Coxs reduces the morphological perturbation of ovarian surface epithelium induced by increased gonadotropins in Wv mice, which model postmenopausal ovarian biology and provides further rationale and support for the use of nonsteroidal anti-inflammatory drugs and specific Cox-1 and/or Cox-2 inhibitors for ovarian cancer prevention in peri- and postmenopausal women. In parallel, we found that in human ovaries, peri- and postmenopausal age is the key determinant of ovarian surface epithelial preneoplastic morphological changes in populations with and without BRCA mutations.[2] Thus, the circumstances in Wv mice are highly pertinent to menopausal women in both biology and pathology. The findings that a reduction instead of complete inhibition of Cox-2 is effective in the suppression of ovarian epithelial lesions and that a compensatory mechanism between the expression of Cox-1 and Cox-2 may offer new strategies for clinical intervention.

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References

1. Gosden RG: Biology of Menopause: The Causes and Consequences of Ovarian Aging. Edited by RG Gosden. New York, Academic Press, 1985
2. Cai KQ, Klein-Szanto A, Karthik D, Edelson M, Daly MB, Ozols RF, Lynch HT, Godwin AK, Xu XX: Age-dependent morphological alterations of human ovaries from populations with and without BRCA mutations. Gynecol Oncol 2006, 103:719–728
3. Nicosia SV: The aging ovary. Med Clin North Am 1987, 71:1–9
4. Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, Boente MP, Lynch HT, Hamilton TC: Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. J Natl Cancer Inst 1996, 88:1810–1820
5. Mintz B: Embryological development of primordial germ-cells in the mouse: influence of a new mutation, Wj. J Embryol Exp Morphol 1957, 5:396–406
6. Dubreuil P, Rottapel R, Reith AD, Forrester L, Bernstein A: The mouse W/c-kit locus. A mammalian gene that controls the development of three distinct cell lineages. Ann NY Acad Sci 1990, 599:58–65
7. Nocka K, Tan JC, Chiu E, Chu TY, Ray P, Traktman P, Besmer P: Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: w37, Wv, W41 and W. EMBO J 1990, 9:1805–1813
8. Reith AD, Rottapel R, Giddens E, Brady C, Forrester L, Bernstein A: W mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit receptor. Genes Dev 1990, 4:390–400
9. Murphy ED: Hyperplastic and early neoplastic changes in the ovaries of mice after genic deletion of germ cells. J Natl Cancer Inst 1972, 48:1283–1295
10. Eldridge JC, McPherson III JC, Mahesh VB: Maturation of the negative feedback control of gonadotropin secretion in the female rat. Endocrinology 1974, 94:1536–1540
11. Murphy ED, Beamer WG: Plasma gonadotropin levels during early stages of ovarian tumorigenesis in mice of the W x/W u genotype. Cancer Res 1973, 33:721–723
12. Blaakaer J, Baeksted M, Micic S, Albrectsen P, Rygaard J, Bock J: Gonadotropin-releasing hormone agonist suppression of ovarian tumorigenesis in mice of the Wx/Wv genotype. Biol Reprod 1995, 53:775–779
13. Cramer DW, Welch WR: Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. J Natl Cancer Inst 1983, 71:717–721
14. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK: Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 1997, 91:197–208
15. Davis BJ, Lennard DE, Lee CA, Tiano HF, Morham SG, Wetsel WC, Langenbach R: Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta. Endocrinology 1999, 140:2685–2695
16. Reese J, Zhao X, Ma WG, Brown N, Maziasz TJ, Dey SK: Comparative analysis of pharmacologic and/or genetic disruption of cyclooxygenase-1 and cyclooxygenase-2 function in female reproduction in mice. Endocrinology 2001, 142:3198–3206
17. Richards JS, Russell DL, Ochsner S, Espey LL: Ovulation: new dimensions and new regulators of the inflammatory-like response. Annu Rev Physiol 2002, 64:69–92
18. Williams CS, Mann M, DuBois RN: The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 1999, 18:7908–7916
19. Prescott SM, Fitzpatrick FA: Cyclooxygenase-2 and carcinogenesis. Biochim Biophys Acta 2000, 1470:M69–M78
20. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM: Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996, 87:803–809
21. Lal G, Ash C, Hay K, Redston M, Kwong E, Hancock B, Mak T, Kargman S, Evans JF, Gallinger S: Suppression of intestinal polyps in Msh2-deficient and non-Msh2-deficient multiple intestinal neoplasia mice by a specific cyclooxygenase-2 inhibitor and by a dual cyclooxygenase-1/2 inhibitor. Cancer Res 2001, 61:6131–6136
22. Chulada PC, Thompson MB, Mahler JF, Doyle CM, Gaul BW, Lee C, Tiano HF, Morham SG, Smithies O, Langenbach R: Genetic disrup-

tion of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. Cancer Res 2000, 60:4705–4708
23. Subbaramaiah K, Dannenberg A: Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol Sci 2003, 24:96–102
24. Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B: The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 2000, 342:1946–1952
25. Cramer D, Harlow B, Titus-Ernstaff L, Bohlke K, Welch WR, Greenberg ER: Over-the-counter analgesics and risk of ovarian cancer. Lancet 1998, 351:104–107
26. Sørensen HT, Friis S, Norgard B, Mellemkjaer L, Blot WJ, McLaughlin JK, Ekbom A, Baron JA: Risk of cancer in a large cohort of nonaspirin NSAID users: a population-based study. Br J Cancer 2003, 88:1687–1692
27. Schildkraut JM, Moorman PG, Halabi S, Calingaert B, Marks JR, Berchuck A: Analgesic drug use and risk of ovarian cancer. Epidemiology 2006, 17:104–107
28. Smith ER, Daly MB, Xu XX: A mechanism for Cox-2 inhibitor anti-inflammatory activity in chemoprevention of epithelial cancers. Cancer Epidemiol Biomarkers Prev 2004, 13:144–145
29. Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA, Smithies O: Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell 1995, 83:473–482
30. Smith WL, Langenbach R: Why there are two cyclooxygenase isozymes. J Clin Invest 2001, 107:1491–1495
31. Gupta RA, Tejada LV, Tong BJ, Das SK, Morrow JD, Dey SK, DuBois RN: Cyclooxygenase-1 is overexpressed and promotes angiogenic growth factor production in ovarian cancer. Cancer Res 2003, 63:906–911
32. Daikoku T, Wang D, Tranguch S, Morrow JD, Orsulic S, DuBois RN, Dey SK: Cyclooxygenase-1 is a potential target for prevention and treatment of ovarian epithelial cancer. Cancer Res 2005, 65:3735–3744
33. Daikoku T, Tranguch S, Trofimova IN, Dinulescu DM, Jacks T, Nikitin AY, Connolly DC, Dey SK: Cyclooxygenase-1 is overexpressed in multiple genetically engineered mouse models of epithelial ovarian cancer. Cancer Res 2006, 66:2527–2531
34. Balkwill F, Coussens LM: Cancer: an inflammatory link. Nature 2004, 431:405–406
35. Clevers H: At the crossroads of inflammation and cancer. Cell 2004, 118:671–674
36. Tsafriri A, Reich R: Molecular aspects of mammalian ovulation. Exp Clin Endocrinol Diabetes 1999, 107:1–11
37. Ozols RF, Bookman MA, Connolly DC, Daly MB, Godwin AK, Schilder RJ, Xu XX, Hamilton TC: Focus on epithelial ovarian cancer. Cancer Cell 2004, 5:19–24
38. Orsulic S, Li Y, Soslow RA, Vitale-Cross LA, Gutkind JS, Varmus HE: Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. Cancer Cell 2002, 1:53–62
39. Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC, Hamilton TC: Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. Cancer Res 2003, 63:1389–1397
40. Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN, Nikitin AY: Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. Cancer Res 2003, 63:3459–3463
41. Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, Jacks T: Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. Nat Med 2005, 11:63–70
42. Chen CM, Chang JL, Behringer RR: Tumor formation in p53 mutant ovaries transplanted into wild-type female hosts. Oncogene 2004, 23:7722–7725
43. Kirtikara K, Morham SG, Raghow R, Laulederkind SJ, Kanekura T, Goorha S, Ballou LR: Compensatory prostaglandin E2 biosynthesis in cyclooxygenase 1 or 2 null cells. J Exp Med 1998, 187:517–523
44. Wang H, Ma WG, Tejada L, Zhang H, Morrow JD, Das SK, Dey SK: Rescue of female infertility from the loss of cyclooxygenase-2 by compensatory up-regulation of cyclooxygenase-1 is a function of genetic makeup. J Biol Chem 2004, 279:10649–10658